

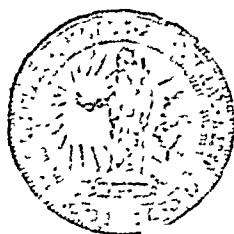


# THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY  
SIMON FLEXNER, M.D.      PEYTON ROUS, M.D.

VOLUME XLVI, No. 1

JULY 1, 1927



COPYRIGHT, 1927, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.  
Authorized for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.  
Authorized June 22, 1918.

*Made in the United States of America*



# CONTENTS

DU NOÛY, P. LECOMTE. Surface tension of serum. XV. The thickness of the monolayer of rabbit plasma.....	1
ROWNTRIE, LEONARD G., and SHONOYA, TAKUJI. Studies in experimental extracorporeal thrombosis. I. A method for the direct observation of extracorporeal thrombus formation. ....	7
SHONOYA, TAKUJI. Studies in experimental extracorporeal thrombosis. II. Thrombus formation in normal blood in the extracorporeal vascular loop. Plate I.....	13
SHONOYA, TAKUJI. Studies in experimental extracorporeal thrombosis. III. Effects of certain anticoagulants (heparin and hirudin) on extracorporeal thrombosis and on the mechanism of thrombus formation. Plates 2 and 3.....	19
MOISE, THEODORE S., and SMITH, ARTHUR H. Diet and tissue growth. V. The effect of dietary protein on the remaining kidney of adult white rats following a unilateral nephrectomy. Plates 4 and 5.....	27
WILBUR, BLAKE C., DALAND, GENIVA A., and COHEN, JOHN. Cell respiration studies. I. A microcalorimeter for the continuous study of the oxygen absorption by living cells.....	43
DALAND, GENIVA A., and ISAACS, RAHAEL. Cell respiration studies. II. A comparative study of the oxygen consumption of blood from normal individuals and patients with increased leucocyte counts (leukemia; chronic myelogenous leukemia).....	53
PEARCE, LOUISE, and RIVERS, THOMAS M. Effect of host immunity to a filterable virus (Virus III) on the growth and malignancy of a transplantable rabbit neoplasm.....	65
PEARCE, LOUISE, and RIVERS, THOMAS M. Effect of a filterable virus (Virus III) on the growth and malignancy of a transplantable neoplasm of the rabbit.....	81
GASPARI, EMILIO L., FLEMING, WILLIAM L., and NEILL, JAMES M. Immunological properties of a typical (S-producing) and a degraded (non-S-producing) strain of Type II pneumococcus with special reference to protective antibodies.....	101
NEILL, JAMES M., and GASPARI, EMILIO L. Factors involved in the infection of mice after vaccination with Type II pneumococci.....	113
SMITH, THEODORE, and LITTLE, RALPH B. Studies on pathogenic <i>B. coli</i> from bovine sources. I. The pathogenic action of culture filtrates.....	123
SMITH, THEODORE, and BEVANT, GLADYS. Studies on pathogenic <i>B. coli</i> from bovine sources. II. Mutations and their immunological significance. Plates 6 and 7.....	133
SMITH, THEODORE. Studies on pathogenic <i>B. coli</i> from bovine sources. III. Normal and serologically induced resistance to <i>B. coli</i> and its mutant.....	141
SMITH, DOROTHEA E. Studies on pathogenic <i>B. coli</i> from bovine sources. IV. A biochemical study of the capsular substance.....	155
MUDD, STUART, and MUDD, EMILY B. H. The surface composition of the tubercle bacillus and other acid-fast bacteria.....	167
MUDD, STUART, and MUDD, EMILY B. H. On the mechanism of the serum sensitization of acid-fast bacteria.....	173
LEVINT, P. A., LANDSTAMME, K., and VAN DER SCHEER, J. Immunization experiments with lecithin.....	197

*The Journal of Experimental Medicine* is issued monthly, two volumes appearing in a year. During the last few years, each volume has contained more than 700 pages and many plates and text-figures.

An INDEX for Volumes I to XX and one for Volumes XXI to XL have been published.

Contributions should be sent to the editors of *The Journal of Experimental Medicine*, Avenue A and 66th Street, New York, N. Y. They should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Articles which exceed in length twenty-five printed pages will be returned to the authors in order that their contents may be reduced to this maximum. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

Beginning with Volume XLI, the subscription price per year (two volumes) is \$10.00, payable in advance. Single copies cost \$1.00. The price of the earlier volumes per year (two volumes) is \$5.00; single copies, 75 cents. Subscriptions will not be accepted to begin in the middle of a volume. With Volume XLI a commission to agents of 10 per cent will be allowed on all subscriptions. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

For information regarding complete sets and back numbers of *The Journal of Experimental Medicine*, application should be made to the Division of Publication of The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.





# CONTENTS

PRAFCE, LOUIS, and MURPHY, JAMES B. Further observations on the inability to transmit a rabbit neoplasm by cell-free materials .....	205
LANDSTEINER, K., and LEVINE, PHILIP. On a specific substance of the cholera vibrio .....	213
CHESLEY, ALAN M., HALL, Y., CHARLES R. L., and KEMP, JAPOLD E. Studies in experimental syphilis. VII. Reinoculation of treated and untreated syphilitic rabbits with heterologous strains of <i>Treponema pallidum</i> .....	223
ROBERTSON, OSWALD H., and SIA, RICHARD H. P. Studies on pneumococcus growth inhibition. VII. The relation of opsonins to natural resistance against pneumococcus infection .....	239
NEILL, JAMES M., and FLEMING, WILLIAM L. Studies on the oxidation and reduction of immunological substances. VI. The "reactivation" of the bacteriolytic activity of oxidized pneumococcus extracts .....	263
FLEMING, WILLIAM L. Studies on the oxidation and reduction of immunological substances. VII. The differentiation of tetanolyisin and tetanospasmin ..	279
JONES, F. S. The effect of heat on antibodies .....	291
JONES, F. S. Agglutination by precipitin .. .. .	303
DOAN, CHARLES A., and SABIN, FLORENCE R. Local progression with spontaneous regression of tuberculosis in the bone marrow of rabbits, correlated with a transitory anemia and leucopenia after intravenous inoculation. Plates 8 to 10.....	315
TILLITT, WILLIAM S. Studies on immunity to <i>Pneumococcus mucosus</i> (Type III). III. Increased resistance to Type III infection induced in rabbits by immunization with R and S forms of pneumococcus .....	343
ZINSSEP, HANS, and TANG, FLI-FANG. Studies in ultrafiltration .....	357
DUVAL, CHARLES W., and HIBBARD, R. J. Nature of the toxic moiety of <i>Streptococcus scarlatinae</i> .....	379

*The Journal of Experimental Medicine* is issued monthly, two volumes appearing in a year. During the last few years, each volume has contained more than 700 pages and many plates and text-figures. An INDEX for Volumes I to XX and one for Volumes XXI to XL have been published.

Contributions should be sent to the editors of *The Journal of Experimental Medicine*, Avenue A and 66th Street, New York, N. Y. They should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Articles which exceed in length twenty-five printed pages will be returned to the authors in order that their contents may be reduced to this maximum. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

Beginning with Volume XLI, the subscription price per year (two volumes) is \$10.00, payable in advance. Single copies cost \$1.00. The price of the earlier volumes per year (two volumes) is \$5.00; single copies, 75 cents. Subscriptions will not be accepted to begin in the middle of a volume. With Volume XLI a commission to agents of 10 per cent will be allowed on all subscriptions. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 65th Street, New York, N. Y.

For information regarding complete sets and back numbers of *The Journal of Experimental Medicine*, application should be made to the Division of Publication of The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.



## CONTENTS

---

STEWART, FRED W. Local specific therapy of experimental pneumococcal meningitis. I. Experimental pneumococcal meningitis in rabbits. Plates 11 to 15.....	391
STEWART, FRED W. Local specific therapy of experimental pneumococcal meningitis. II. The production, pathology, and treatment of Type I pneumococcal meningitis in dogs. Plates 16 to 21.....	409
MACKAY, EATON M., and MACKAY, LOIS LOCKARD. Enlargement of the adrenal cortex in experimental uremia. Plates 22 and 23.....	429
DOMINGUEZ, R. The systolic blood pressure of the normal rabbit measured by a slightly modified Van Leersum method. Final report.....	443
DOMINGUEZ, R. Experimental atherosclerosis and blood pressure in the rabbit.	463
DOCHET, A. R., and STEVENS, FRANKLIN A. Studies on the biology of streptococcus. VII. Allergic reactions with strains from erysipelas.....	487
SHWARTZMAN, GREGORY. Studies on streptococcus bacteriophage. I. A powerful lytic principle against hemolytic streptococci of erysipelas origin....	497
DOAN, CHARLES A., and ZERFAS, LEON G. The rhythmic range of the white blood cells in human, pathological leucopenic and leucocytic states, with a study of thirty-two human bone marrows.....	511

---

*The Journal of Experimental Medicine* is issued monthly, two volumes appearing in a year. During the last few years, each volume has contained more than 700 pages and many plates and text-figures. An INDEX for Volumes I to XX and one for Volumes XXI to XL have been published.

Contributions should be sent to the editors of *The Journal of Experimental Medicine*, Avenue A and 66th Street, New York, N. Y. They should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Articles which exceed in length twenty-five printed pages will be returned to the authors in order that their contents may be reduced to this maximum. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

Beginning with Volume XLI, the subscription price per year (two volumes) is \$10.00, payable in advance. Single copies cost \$1.00. The price of the earlier volumes per year (two volumes) is \$5.00; single copies, 75 cents. Subscriptions will not be accepted to begin in the middle of a volume. With Volume XLI a commission to agents of 10 per cent will be allowed on all subscriptions. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

For information regarding complete sets and back numbers of *The Journal of Experimental Medicine*, application should be made to the Division of Publication of The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

# MONOGRAPHS—Continued.

- No. 2. WILLIAM TRAVIS HOWARD and O. CARL T. SCHULTZ. Studies in the biology of tumor cells. Plates 1-6. (Issued February 15, 1911.) Price, \$1.00.
- No. 3. BENJAMIN T. TERRY. Chemo-therapeutic trypanosome studies with special reference to the immunity following cure. (Issued March 15, 1911.) Price, \$1.00.
- No. 4. FRANCIS W. PEABODY, GEORGE DEAMER, and A. E. DEWEER. A clinical study of acute polymycolitis. Plates 1-13. (Issued June 24, 1912.) Price, \$1.00.
- \*No. 5. J. P. SIMONDS. Studies in *Bacillus subtilis* with special reference to clarification and to its relation to diarrhea. (Issued September 27, 1915.) Price, \$1.00.
- \*No. 6. JAMES L. STODDARD and ELLIOTT C. CUTLER. Tetanus infection in man. Plates 1-9. (Issued January 31, 1916.) Price, \$1.00.
- No. 7. OSWALD T. AVERY, H. T. CHICKERING, ELLER COLE, and A. E. DEWEER. Acute lobar pneumonia. Prevention and serum treatment. Plates 1-3. (Issued October 16, 1917.) Price, \$1.00.
- \*No. 8. ERNEST C. DICKSON. Botulism. A clinical and experimental study. Plates 1-10. (Issued July 31, 1918.) Price, \$1.00.
- \*No. 9. JAMES HOWARD BROWN. The use of blood agar for the study of streptococci. Plates 1-31. (Issued January 21, 1919.) Price, \$2.00.
- No. 10. WILLIAM G. MACCALLUM. The pathology of the pneumonia in the United States Army camps during the winter of 1917-18. Plates 1-53. (Issued April 16, 1919.) Price, \$1.50.
- No. 11. FREDERICK M. ALLEN, EDGAR STILLMAN, and RICHARD PRIZ. Total dietary regulation in the treatment of diabetes. 62 charts. (Issued October 15, 1919.) Price, \$4.50.
- No. 12. LEWIS H. WEED, PAUL WEGENORTH, JAMES B. AYER, and LLOYD D. FRISON. A study of experimental meningitis. A series of papers from the Army Neuro-Surgical Laboratory. Plates 1-17. (Issued March 25, 1920.) Price, \$1.00.
- No. 13. WERNER MARCHAND. The early stages of Tabanids (horse-flies). Plates 1-15. (Issued November 15, 1920.) Price, \$2.00.
- No. 14. S. T. DARLING and W. G. SMILLIE. Studies on hookworm infection in Brazil. First paper. (Issued February 1, 1921.) Published for the International Health Board.
- No. 15. I. J. KLIIGLER. Investigation on soil pollution and the relation of the various types of privies to the spread of intestinal infections. (Issued October 10, 1921.) Published for the International Health Board.
- No. 16. ABRAHAM L. GARBAT. Typhoid carriers and typhoid immunity. (Issued May 10, 1922.) Price, \$1.25.
- No. 17. WILSON G. SMILLIE. Studies on hookworm infection in Brazil. Second paper. (Issued May 12, 1922.) Published for the International Health Board.
- No. 18. P. A. LEVENE. Hexosamines, their derivatives, and mucins and mucoids. (Issued July 7, 1922.) Price, \$1.25.
- No. 19. THEOBALD SMITH and RALPH B. LITTLE. Studies in vaccinal immunity towards disease of the bovine placenta due to *Bacillus abortus* (infectious abortion). (Issued November 15, 1923.) Price, \$1.25.
- No. 20. HIDEYO NOGUCHI *et al.* Experimental studies of yellow fever in northern Brazil. Plates 1-5. (Issued August 9, 1924.) Published for the International Health Board.
- No. 21. JAMES B. MURPHY. The lymphocyte in resistance to tissue grafting, malignant disease, and tuberculous infection. An experimental study. Plates 1-20. (Issued September 20, 1926.) Price, \$2.00.
- No. 22. HARVEY CUSHING and LEO M. DAVIDOFF. The pathological findings in four autopsied cases of acromegaly with a discussion of their significance. 104 half-tones in the text. (Issued April 23, 1927.) Price, \$2.00.

## SURFACE TENSION OF SERUM.

### XV. THE THICKNESS OF THE MONOLAYER OF RABBIT PLASMA.

By P. LECOMTE DU NOÛY, Sc.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 5, 1927.)

In the preceding papers of this series,<sup>1</sup> it has been shown that a well defined minimum value of the static surface tension of fresh, clear serum could be observed at a given high dilution, and the conclusion was reached that under given conditions serum and serum solutions behave as true solutions. In order to explain the presence of this minimum, —or of these minima, as in most cases more than one can be observed, —it was necessary to resort to the hypothesis that all the constituents of the serum acted as though they were bound together in the shape of a huge molecule and that at some critical concentrations, the organization of these molecules in the surface layer decreased the value of the tension measured by the du Noüy tensiometer. The similarity of this phenomenon with that observed in the case of sodium oleate,<sup>2</sup> egg albumin,<sup>3</sup> and recently digitonin,<sup>4</sup> the important decrease in the rate of evaporation at the same critical concentration,<sup>5</sup> and the displacement of the minimum when the surface of adsorption was changed,<sup>6</sup> seemed to bear out this hypothesis satisfactorily.

The question then arose as to whether solutions of plasma would behave in the same way, namely, as large molecules capable of organizing themselves at a given concentration, or whether they would behave as though molecules of another substance but of approximately the

<sup>1</sup> du Noüy, P. L., Surface tension of serum, Papers I-X, *J. Exp. Med.*, 1922, xxxv, to 1924, xl; Paper XI, *J. Gen. Physiol.*, 1924, vi, 625; Papers XII-XIV, *J. Exp. Med.*, 1925, xli to xlii.

<sup>2</sup> du Noüy, P. L., *Phil. Mag.*, 1924, xlviii, 264, 664.

<sup>3</sup> du Noüy, P. L., *J. Biol. Chem.*, 1925, lxiv, 595.

<sup>4</sup> Unpublished experiments.

<sup>5</sup> du Noüy, P. L., *J. Exp. Med.*, 1924, xxxix, 717.

<sup>6</sup> du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 133.

same molecular weight were added to the serum. In other words, when fibrinogen is present in the serum, does it exist separately, or is it part of the serum molecule, which would then be a "plasma molecule"?

If the fibrinogen is supposed to exist as a separate substance in solution (plasma = serum molecules + fibrinogen molecule), then the concentration at which the principal minimum of surface tension occurs will not be changed as the space occupied in the horizontal plane by the "serum molecules" will be the same. By diluting serum or plasma 10,500 times, a minimum should be expected in both cases, although the concentration of the latter in proteins would be greater, and consequently also the thickness of the adsorbed layer. The same would happen if the fibrinogen were bound at one end of the serum molecule, without involving any change in structure. Therefore, in case no shift should be observed, no definite answer could be given as to whether the fibrinogen exists separately or whether it is merely an easily detachable group fixed at one end of the serum molecule.

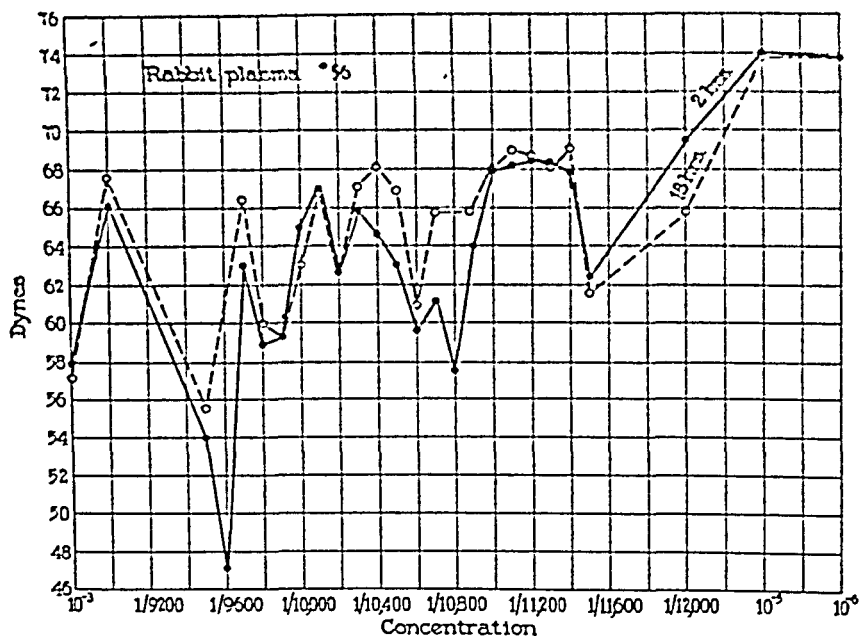
But if the fibrinogen, instead of being merely additive, were actually part of the molecule, the whole structure and symmetry of the assumed "plasma molecule" might be different from that of the "serum molecule," and consequently, the space occupied in the horizontal plane by these two molecules might also differ. If it is assumed that the "plasma molecule" can orient itself in a monolayer as does the "serum molecule," a shift in the place of the minimum is to be expected. Therefore, should a minimum be observed with plasma solutions, and should this minimum occur at the same concentration as it does with serum, it would indicate that the orientation and dimensions of the serum molecules in the horizontal plane are unchanged. In this case, no answer could be given as to the possible link between fibrinogen and serum; on the contrary, should the principal minimum be shifted toward higher or lower concentrations, one could logically assume that the molecules present in the solution are different in size and shape from those of the serum, but similarly capable of organizing as polarized units.

#### EXPERIMENTAL AND RESULTS.

The blood was collected and centrifuged immediately, and the plasma was diluted as soon as possible in saline solution. In a few

cases, coagulation occurred at the concentration 1:100, but never at 1:1000. When dilutions were made rapidly, no difficulty was encountered. The experimental part of this work was carried out by Mr. J. Zwick. (Text-figs. 1 and 2.)

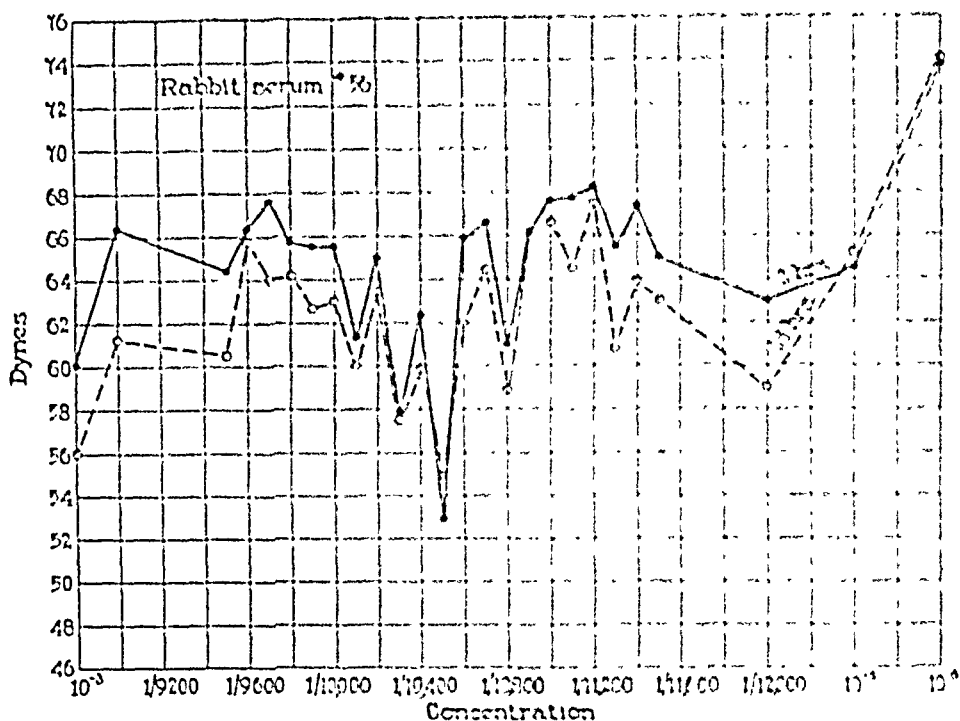
The results of twenty-two series of measurements are not as sharply defined as one might have hoped. However, when the frequency of occurrence of the minima at a given concentration is taken into consideration (Text-fig. 3), it is clear that minima are observed in the case of



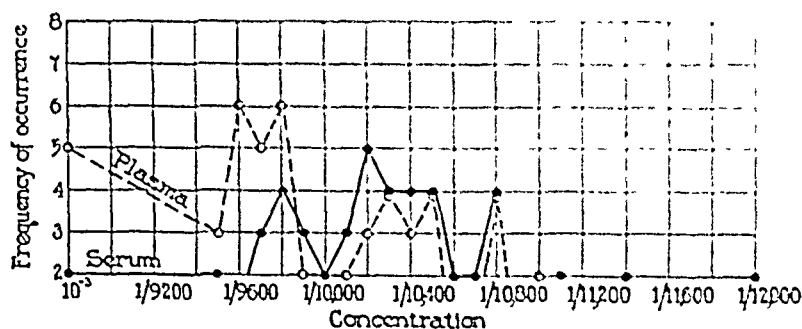
TEXT-FIG. 1. Experiment 1. Static values of diluted rabbit plasma, as a function of the concentration. The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.

plasma and that the most important of them are shifted with respect to those of the serum. Text-fig. 4 expresses the mean value of the twenty-two curves. If the minima which only occurred twice or less are omitted as due to experimental errors, it is obvious from Text-fig. 3 that out of thirty-eight minima observed in the eleven series of experiments with plasma, twenty or 53 per cent occurred at concentrations between 1:9500 and 1:9800. Out of the thirty-four minima found for





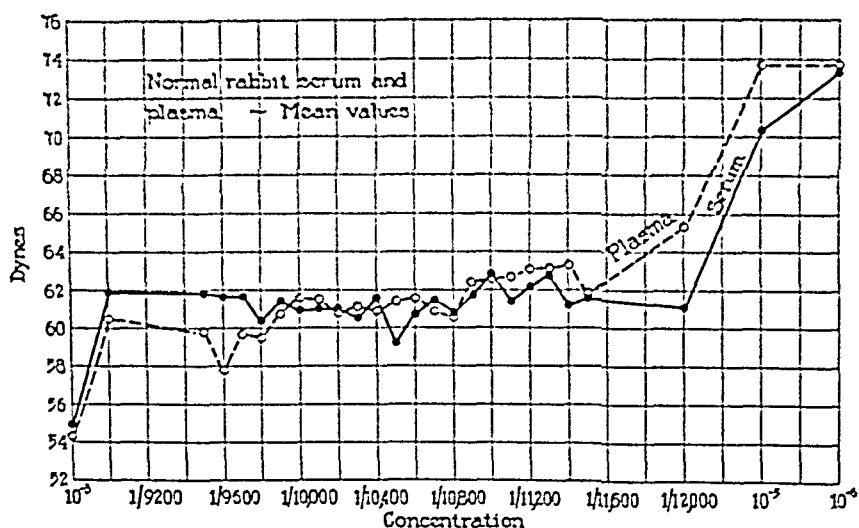
TEXT-FIG. 2. Experiment 2. Static values of diluted rabbit serum, as a function of the concentration. The two curves give the results of two series of measurements, one after 2 hours, the other after 18 hours.



TEXT-FIG. 3. Frequency of occurrence of the minima of static surface tension in plasma and serum (eleven experiments for each).

serum under the same conditions, seventeen or 50 per cent occurred at concentrations between 1:10,200 and 1:10,500. Although the minima 1:9600 for plasma and 1:10,500 for serum are quantitatively more important, as is seen from Text-figs. 3 and 4, one must not neglect the fact that the minimum 1:9800 (plasma) shows the same

degree of probability (Text-fig. 3), and that the minimum 1:10,200 (serum) was even more frequent than that at 1:10,500. Therefore, it seems desirable to take into account the mean values, namely, 1:9700 for plasma, and 1:10,350 for serum. These concentrations differ by about 6.3 per cent. This percentage expresses the difference in thickness of monolayers of plasma and of serum. It is assumed that the specific gravity of fibrinogen is the same as that of the other proteins of the serum, which is probably very nearly true. Hence, the thickness of the plasma monolayer is greater by 6.3 per cent in



TEXT-FIG. 4. Mean values of eleven series of measurements for serum and eleven series of measurements for plasma.

round figures than that of serum. As the "length" of the total "serum molecule," according to our former determinations,<sup>5</sup> is about 40.5 Ångström units, or 4.05m $\mu$  the length of the "plasma molecule" will be 43.05 Ångströms in round figures, or 4.3m $\mu$ . This difference should correspond to the amount of fibrinogen present. Two careful determinations of the fibrin content of rabbit plasma, with two different methods,<sup>7</sup> gave the figures 4.5 and 4.65 per cent of total proteins.

<sup>7</sup> Van Slyke's method, and the method consisting of weighing the dry substances of the plasma and of the serum, after dialyzing the salts out, then the fibrin, proper care being taken to remove all the salts by washing the fibrin carefully.

According to Lambling,<sup>8</sup> only 60 to 70 per cent of the fibrinogen is transformed into fibrin after coagulation. If 65 per cent is taken as a mean value, 4.6 per cent of fibrin corresponds to about 7 per cent of fibrinogen. The shift in the minimum, therefore, seems to be in good accord with the fibrinogen content, rather than with the fibrin content. However, it is not the intention of the writer to insist upon his figure as being a check of Lambling's statement, as too little is known at present about this subject. The error introduced through the fact that the amount of fibrinogen is expressed in per cent of the proteins and that the increase in thickness of the monolayer is expressed in per cent of total serum does not amount to more than about 0.3 per cent and is negligible in consideration of the other causes of error.

#### CONCLUSIONS AND SUMMARY.

Experiments are reported which indicate that a shift toward higher concentrations is observed in the minimum value of the static surface tension when plasma instead of serum solutions is used. The amount of the shift, expressed as a function of the concentration, shows that the figures are in satisfactory agreement with the determined amount of fibrinogen in the plasma.

Some evidence is given that "plasma molecules" capable of organizing themselves on adsorbing surfaces exist in plasma, and that their length would be approximately 4.3  $\mu$  in round figures, instead of 4.0  $\mu$  for the serum. The area occupied in the plane of adsorption by one individual molecule is, however, smaller than that occupied by the "serum molecule," thus indicating a marked structural difference between the two, the "plasma molecule" being narrower but longer than the "serum molecule." This difference may be due either to a different orientation accompanied by an increase in one of the dimensions, or else to an actual difference in structure with respect to the main axis, resulting in a decrease in the mean diameter of the "serum molecule" with an increase in the length of its main axis. The mass of the "plasma molecule" is about 6.3 per cent larger than that of the "serum molecule," in the case of rabbit serum.

<sup>8</sup> Lambling, E., *Précis de biochimie*, Paris, 1911, 250.

# STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

## I. A METHOD FOR THE DIRECT OBSERVATION OF EXTRACORPOREAL THROMBUS FORMATION.

BY LEONARD G. ROWNTREE, M.D., AND TAKUJI SHIONOYA.\*

*(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)*

(Received for publication, April 6, 1927.)

Pulmonary embolism is one of the problems still a challenge to medicine and surgery. In the Mayo Clinic, the necropsy records show that it has been responsible for 7.3 per cent of the postoperative deaths during the last 10 years. Its annual toll throughout the world must be very large, and is probably much greater than is generally accepted, or than most statistics indicate, since pulmonary embolism is readily overlooked at necropsy unless sought for as a routine and specifically. Because of its important bearing on surgical mortality (2-11) we are making a study of thrombosis with the hope that eventually some means may be found whereby postoperative pulmonary embolism may be avoided or prevented.

In connection with this problem, one of us recalls some earlier studies carried on with John J. Abel, in which a dialyzing apparatus or an artificial kidney was used for the removal of diffusible substances from the circulating blood of living animals. In those experiments it was necessary to combat thrombosis constantly despite the fact that hirudin was employed in generous quantities as an anticoagulant. From this experience, it was thought that a similar, but simpler, apparatus might be employed advantageously in the study of the various stages of formation of thrombi, particularly that of white thrombi.

\* Fellow of the Rockefeller Foundation.

*Principle of the Method.*

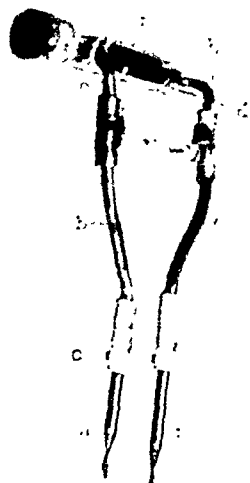
The method is simple: it consists in the introduction into the circulation of an extracorporeal vascular loop. By means of a cannula, the artery of the animal is attached to a collodion dialyzing tube, immersed in physiologic solution of sodium chloride, or other form of solution, and the blood is returned to the animal's body by another cannula attached to the vein. Before the instrument is attached to the vessels, the tubes and cannulas are completely filled with a solution of sodium chloride which approximates in its salt content the plasma of the animal, and this fluid is displaced into the body when the circulation through the apparatus is established.

During the experiment, the blood flows in a perfectly enclosed system and is returned to the body within a few seconds without having been exposed to contact with the air. On the way, it passes through the collodion tube which permits of an interchange of diffusible substances between the blood and the fluids in the outside container.

Since the inner surface of the apparatus is a foreign surface, coagulation occurs rapidly in the normal animal, within 4 to 10 minutes, but coagulation of the blood may be delayed or prevented by paraffining the tubes or by the previous injection of anticoagulants, such as heparin and hirudin, into the vein. Despite the presence of these anticoagulants, white thrombi are formed and thrombosis eventually occurs. The rate of thrombus formation can be influenced by various substances introduced into the circulation directly, or indirectly, and especially locally by dialysis through the collodion membrane, and the factors affecting each stage of clot formation may thereby be analyzed and assayed. The apparatus lends itself readily to the study of the influence of the mechanical, physical and chemical factors affecting the process of thrombosis. The experiments are carried out under complete trichloro-tertiary-butylalcohol anesthesia and are usually interrupted within 3 or 4 hours. The proportion of blood in the extracorporeal loop (about 2 cc.) is practically negligible so far as the physiology of the circulation of the animal is concerned.

*The Apparatus.*

The apparatus (Text-fig. 1) is simply an artificial circulatory loop permitting of extracorporeal blood flow. It consists of two parts connected by a collodion tube, which is immersed in a tube used as a container for dialyzing solutions. A glass frame holds the apparatus in place. The centrifugal and centripetal parts of the apparatus are attached respectively to the carotid artery and jugular vein. For purposes of handling and cleansing, each part is made in three sections, which are connected by means of rubber tubing. An ordinary arterial glass cannula, *a*, (about 4.5 cm. in length) is attached to the vessel and connected with the side tube *b*, which is about 6 cm. long and curved slightly to facilitate its connection with the corner tube *c* (5 cm.), which is curved in two directions, horizontally to approach the collodion tube *g*, which connects it with the other half of the apparatus, and downward to provide for immersion of the collodion membrane in the solution of the outside container *h*. The side tube and corner tube are paraffined. The collodion tube is attached to the two corner tubes by ligatures. The containing jacket is a short, stoppered test-tube with an opening in the side, which permits the immersion of the collodion tube in the solution of the dialyzing system. When the experiment is in progress, the blood flows from the artery through the cannula, side tube and corner tube of the arterial part into the collodion tube, and then into the corner tube *d*, side tube *e*, and cannula *f*, of the centripetal part back to the veins of the animal.



TEXT-FIG. 1. Extracorporeal loop.

*Technic of the Experiment.*

Rabbits, dogs or cats are suitable for such experiments, but throughout all our experiments rabbits have been employed; compared with dogs, they are more economical in the use of the anticoagulants.

Anesthesia is induced by the intraperitoneal injection of a saturated solution of trichloro tertiary-butylalcohol in olive oil (from 1.5 to 2 cc. for each kilo of body weight). The apparatus is prepared, the clasp side tube and corner tube paraffined, the collodion tube tied in place and the whole apparatus filled with phycolene collum chloride solution.

The cannulas are introduced in the usual way, particular care being taken to produce as little trauma as possible. The vessels are clamped gently and carefully, to preserve, so far as possible, the integrity of the intima. With the cannulas tied in place, the cut wound of the vessels are outside of the circuit, and hence little if any tissue juice or thromboplastic substance finds its way into the circulation in the extracorporeal loop. All the experiments are conducted under practically identical conditions. If anticoagulants are administered, unusual care must be exercised in the isolation and ligation of all vessels. This is necessary in the absence of clotting resulting from the anticoagulants, otherwise large quantities of blood may be lost, even from the smallest vessels.

The carotid artery and jugular vein are clamped until the apparatus is attached and then the artificial circulation is established, the blood flowing from the carotid artery into the tubes and back into the animal through the jugular vein. The blood flow can be seen clearly, particularly at points of constriction and in the neighborhood of curves. Currents, eddies and swirling motion may be obvious, particularly in the cannula where the arterial blood enters, and in the collodion tube. When doubt exists, proof of continued circulation can be readily obtained by clamping off the jugular vein and observing the presence or absence of ballooning of the vessel proximal to the clamp.

Thrombus formation can be directly observed through the collodion wall of the sac. This is more likely to occur in the neighborhood of wrinkles and irregularities in the surface of the collodion tube. Thrombi are visible first as pin-point areas, growing and radiating gradually in a direction counter to the blood stream and may attain considerable size (0.5 by 0.05 cm.). Clotting of the blood or the actual formation of the red thrombus may or may not occur, depending on the conditions of the experiment.

At will, any stage of the thrombus formation may be studied through

direct observation with the tube *in situ* or after the removal of the collodion tube. In the early stages of clotting, after the removal of the tube the surface may be gently washed with sodium chloride solution and observed directly, or studied under the microscope with or without the aid of special stains; or the specimen may be prepared and embedded in paraffin for section staining and ordinary pathologic study. The clots themselves may also be subjected to study.

#### DISCUSSION.

This method lends itself to the study of thrombus formation and the part played by various factors under many varying conditions. Thus the influence of mechanical, physical, physiologic, pharmacologic and pathologic factors may be determined, measured and to a certain extent assayed. Mechanically, several factors influence the rate of coagulation. The absolute and relative size of the tubes employed, the nature of the surfaces exposed, constriction, dilatation or angulation of the channel or changes in hydrostatic pressure resulting from differences in the levels of the blood stream, all influence the rate of blood flow and of coagulation. Physically, the temperature of the fluid surrounding the collodion tube is of importance. Physiologically, the vigor of the circulation and the size of the animal play some rôle. Pharmacologically,<sup>1</sup> drugs may be introduced which affect vigor and rate of circulation, or which directly accelerate or inhibit the process of coagulation. These may be injected intravenously, or diffusible drugs may be introduced into the extracorporeal loop locally by means of dialysis. Pathologic processes may be induced experimentally in animals, such as obstructive jaundice, chronic arsenic or phosphorus poisoning, and the influence of these diseases on the process of thrombosis determined. Finally, many combinations of these various factors may be studied simultaneously such, for instance, as the influence of transfusion or of various thromboplastic substances on the

<sup>1</sup> Since the phenomena of agglutination of platelets and of coagulation of blood are so imperfectly understood it has seemed better to undertake a wide range of experiments even including such as might *a priori* be expected to be fruitless. Results at variance with present hypotheses might help clarify these vexing problems.



process of coagulation in the presence of induced obstructive jaundice, or the influence of calcium on coagulation in a jaundiced or in a heparinized animal. The influence of these various factors is already under investigation and will form the basis of subsequent reports.

#### SUMMARY.

A new method has been described for the study *in vivo* of thrombus formation in blood circulating extracorporeally in an artificial loop. This method permits of control of many factors, and hence of intensive study of thrombus formation under many and varied conditions. It admits of separate study of the formation of white thrombi and of the deposition of fibrin, which may apparently be independent processes.

#### BIBLIOGRAPHY.

1. Abel, J. J., Rowntree, L. G., and Turner, B. B., On the removal of diffusible substances from the circulating blood of living animals by dialysis, *J. Pharmacol. and Exp. Therap.*, 1913-14, v, 275.
2. Boland, F. K., Post-operative thrombophlebitis, *South Med. J.*, 1921, xiv, 307.
3. Bondy, O., Ueber das postoperative Verhalten nach Lumbalanästhesie, *Gynäk. Rundschau*, 1910, iv, 99.
4. Cutler, E. C., and Hunt, A. M., Postoperative pulmonary complications, *Arch. Surg.*, 1920, i, 114.
5. Farrar, L. K. P., The incidence of pulmonary embolism and thrombosis following hysterectomy for myoma uteri, *Am. J. Obst. and Gynec.*, 1921, ii, 286.
6. Hampton, H. H., and Wharton, L. R., Venous thrombosis, pulmonary infarction and embolism following gynecological operations, *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 95.
7. Klein, H. V., Die puerperale und postoperative Thrombose und Embolie, *Arch. Gynäk.*, 1911, xciv, 117.
8. de Quervain, F., Relationship between histological structure and biological activeness of goiter tissue, *Surg., Gynec. and Obst.*, 1922, xxxiv, 513.
9. Ranzi, E., Ueber postoperative Lungencomplicationen embolischer Natur, *Arch. klin. Chir.*, 1908, lxxxvii, 380.
10. Wharton, L. R., and Pierson, J. W., The minor forms of pulmonary embolism after abdominal operations, *J. Am. Med. Assn.*, 1922, lxxix, 1904.
11. Wilson, L. B., Fatal post-operative embolism, *Ann. Surg.*, 1912, lvi, 809.

# STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

## II. THROMBUS FORMATION IN NORMAL BLOOD IN THE EXTRACORPOREAL VASCULAR LOOP.

By TAKUJI SHIONOYA.\*

(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)

### PLATE 1.

(Received for publication, April 6, 1927.)

A study of thrombosis necessitates a consideration of the theory of the clotting of blood. In this country, Howell's theory of coagulation is accepted almost exclusively. It is not my desire to enter into controversial matters either in regard to the mechanism of clotting or to the origin and nature of platelets. Howell's theory may be readily recalled from the following schema.

*Coagulation Factors in the Circulating Blood:*

Prothrombin, from platelets.

Antiprothrombin (heparin), from liver.

Calcium.

Fibrinogen.

*The Mechanism of Clotting.*—Cellular elements yield thromboplastic substances.

Thromboplastic substance neutralizes antiprothrombin.

Prothrombin + calcium = thrombin.

Thrombin + fibrinogen = fibrin = clot.

From the following investigations it will be evident that the deposition of platelets and the liberation of fibrin may occur as two distinct though probably related phenomena. By the method employed it is possible to study these factors separately.

The methods employed by Zahn (6), Pitres (3), Bizzozzero (1), Eberth and Schimmelbusch (2, 4) for the study of experimental

\* Fellow of the Rockefeller Foundation.

thrombosis had involved observations on the mesenteric vessels of living animals after the vessels have been injured chemically or mechanically. Welch (5) used dogs chiefly, and produced thrombi of the femoral vessels and jugular vein by various procedures, such as the application of caustics, the introduction of foreign bodies and mechanical injuries. While all these methods offer excellent opportunities for the examination of injuries of very small parts of the vessels, repeated serial sections, especially during the short period of the first stage of thrombosis, are precluded, unless more than one vessel is brought into the study. With the method of investigating extracorporeal thrombosis, described in Paper I of this series, the mode and mechanics of thrombus formation, the structure of thrombi and the effects of mechanical, chemical and physical and pathologic factors on thrombus formation can be readily investigated.

Before discussing in detail the various investigations on factors influencing experimental extracorporeal thrombosis, the formation of thrombi in normal blood in the extracorporeal circulation must be discussed briefly.

#### EXPERIMENTAL STUDIES.

Experiments were performed according to the description in Paper I of this series, and throughout this work the same individual apparatus has been used constantly. As soon as the clamps on the vessels are released, a violent stream of blood enters the apparatus through the arterial cannula, and returns to the jugular vein by means of the venous cannula, after having passed through a collodion dialyzing tube which is immersed in a physiologic solution of sodium chloride at body temperature. Pulsations of the blood stream, eddies, currents and whirls can be easily observed through the glass and collodion walls. This is most striking in the wider part of the arterial cannula, as the stream reaches this point only after passing through the constricted part of the cannula. In the collodion tube also, streaming blood and pulsating movement can be seen before clotting occurs. Even after the latter has taken place, the pulsation of the collodion tube can be seen and felt at times. In 2 or 3 minutes a few white thrombi appear in the constricted part of the venous cannula and in the collodion tube and the blood clots quickly. This occurs also on the foreign surface

of the collodion tube and on the irregular surfaces of connecting parts of the tubing. In the collodion tube, which presents a greater caliber than other parts of the apparatus, the blood current is rather slower, the clotting begins as soon as small collections of platelets appear, and in some instances even begins before white thrombi form. At first, clotting is incomplete and leaves sufficient room for the stream to continue. White thrombi formed in the arterial part of the apparatus are dislodged by the violent current. These may lodge in the collodion tube, or be carried to the venous half of the apparatus and tend to gather especially in the constricted part of the venous cannula. The latter is an important factor in determining the duration of flow. Fibrin is formed around the platelet thrombi and soon the constricted part of the venous cannula is obstructed usually in from 4 to 10 minutes after the circulation is established (Fig. 1). Then the blood which is contained at rest in the venous half of the apparatus clots. The blood current thus becomes gradually slower and the white thrombi stick to the inner surfaces of the arterial cannula also. When complete obstruction occurs, the blood in the apparatus gradually darkens and no more ballooning of the jugular vein can be observed on clamping it below the cannula.

During the period of actual circulation, or after the obstruction is complete, the red thrombus begins to form in the jugular vein following the deposition of white thrombi in the constricted part of the venous cannula. These may grow rapidly, extending in the direction of the circulating blood. Within 30 minutes the red thrombus in the jugular vein may be 0.5 cm. long and as large as the caliber of the jugular vein. The intima of the vein may have been more or less injured by clamping or by insertion of the venous cannula, so that the thrombus may be definitely attached to the wall of the jugular vein. As the apparatus consists of sections, connecting parts leave irregular surfaces and white thrombi are formed; then fibrin forms around the thrombi; thus the mixed thrombus grows into the lumen.

More remarkable is the case in the collodion tube in the transition between the collodion tube and paraffined glass tubes. The white thrombi are laid down in ring form, at the junction of the glass and collodion tubes, where the lumen undergoes marked change. Fibrin forms in 2 or 3 minutes, and later, red clots attach to it. In other

wider parts of the tube, as soon as a few tiny white thrombi are produced, films of blood clot stick to the membrane successively. Thus a few minutes after obstruction, the collodion tube is almost filled with red clot.

In the serial sections stratification is seen in the red clot, that is, the clot is a mural red thrombus, quite flat, with a wide base developed from the tiny white thrombus. When the obstruction occurs very quickly, and the clotting time is very short, or when the blood stream is very slow, no white thrombi can be recognized. When the blood stream is very slow, the constricted part of the arterial cannula may be obstructed by white thrombi and the blood of the whole apparatus comes to standstill and clots as a column. White thrombi occasionally form in the carotid artery, starting from the injured intima or from the white thrombi in the constricted part of the arterial cannula.

Two of the seven normal rabbits weighed 2500 gm. each and presented large arteries and an unusually vigorous stream, so that circulation in the apparatus persisted for 30 minutes. In these two instances many rather large white thrombi appeared in almost all parts of the collodion tube and the venous part of the apparatus. While the vigor of the stream tends to prevent platelets from adhering to the abnormal surfaces of the apparatus and to prevent fibrin from being deposited on the surfaces of white thrombi, the stream in the venous cannula, on the other hand, is not powerful enough to wash away the white thrombi gathered there. The collodion tube in these animals presented a thin layer of clot at its bottom, and ring forms of large mixed thrombi at the points of connection. But true obstruction takes place always in the constricted parts of the cannulas sooner or later, owing to the mixed thrombi formed either in the venous or in the arterial cannula, the exact site depending largely on the vigor of the blood stream.

#### SUMMARY.

Thrombus formation has been studied in normal rabbits with an experimental method of establishing extracorporeal circulation. In the normal animal circulation in the extracorporeal loop usually ceases in from 6 to 10 minutes, or at most, in very large and vigorous

animals, in 25 minutes.<sup>1</sup> Cessation of the circulation is due most frequently to obstruction of the venous cannula (sometimes of the arterial cannula) by a mass of white thrombi and secondary fibrin formation around it. The site of the clot is determined somewhat by the swiftness of the blood stream.

In the collodion tube red mural thrombi are obtainable as a rule. They are flat and present a wide base resting on tiny white thrombi. After the complete obstruction of the circulation, the blood in the apparatus clots very rapidly. When the obstruction occurs very quickly either in the arterial or venous cannula, or when the blood stream is very slow, the clotting in the loop may occur before platelets are laid down in large numbers or before the formation of the white thrombi is evident.

Mixed thrombi are found in the jugular vein, and have their inception in the white thrombi, in the cannula or from the injured intima. They extend in the direction of the blood flow.

#### BIBLIOGRAPHY.

1. Bizzozero, J.. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung, *Arch. path. Anat. u. Physiol.*, 1882, xc, 261.
2. Eberth, J. C., and Schimmelbusch, C., Experimentelle Untersuchungen über Thrombose, *Arch. path. Anat. u. Physiol.*, 1886, ciii, 39.
3. Pitres, A., Recherches expérimentales sur le mode de formation et sur la structure des caillots qui déterminent l'hémostase, *Arch. physiol. norm. et path.*, 1876, iii, series 2, 230.
4. Schimmelbusch, C., Die Blutplättchen und die Blutgerinnung, *Arch. path. Anat. u. Physiol.*, 1885, ci, 201.
5. Welch, W. H., The structure of white thrombi. Papers and addresses, Baltimore, 1920, i, 47.
6. Zahn, F. W., Untersuchungen über Thrombose, *Arch. path. Anat. u. Physiol.*, 1875, lxii, 81.

#### EXPLANATION OF PLATE 1.

FIG. 1. White thrombus mass with red clot in the venous cannula (in the case of normal blood at the end of 10 minutes). *a*, mass of white thrombi. *b*, red clot.  $\times 100$ .

<sup>1</sup> This is true when only the side and corner tubes are paraffined. When all glass tubes are paraffined, circulation is maintained for a number of hours, as will appear in a future communication.



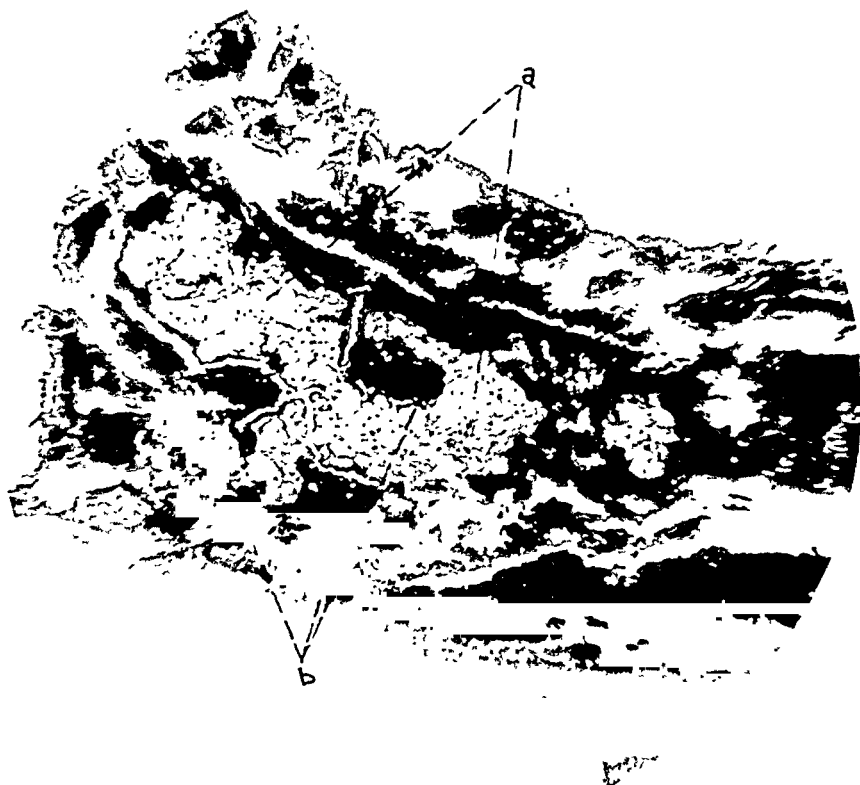


FIG. 1.





# STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

## III. EFFECTS OF CERTAIN ANTICOAGULANTS (HEPARIN AND HIRUDIN) ON EXTRACORPOREAL THROMBOSIS AND ON THE MECHANISM OF THROMBUS FORMATION.

By TAKUJI SHIONOYA.\*

*(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)*

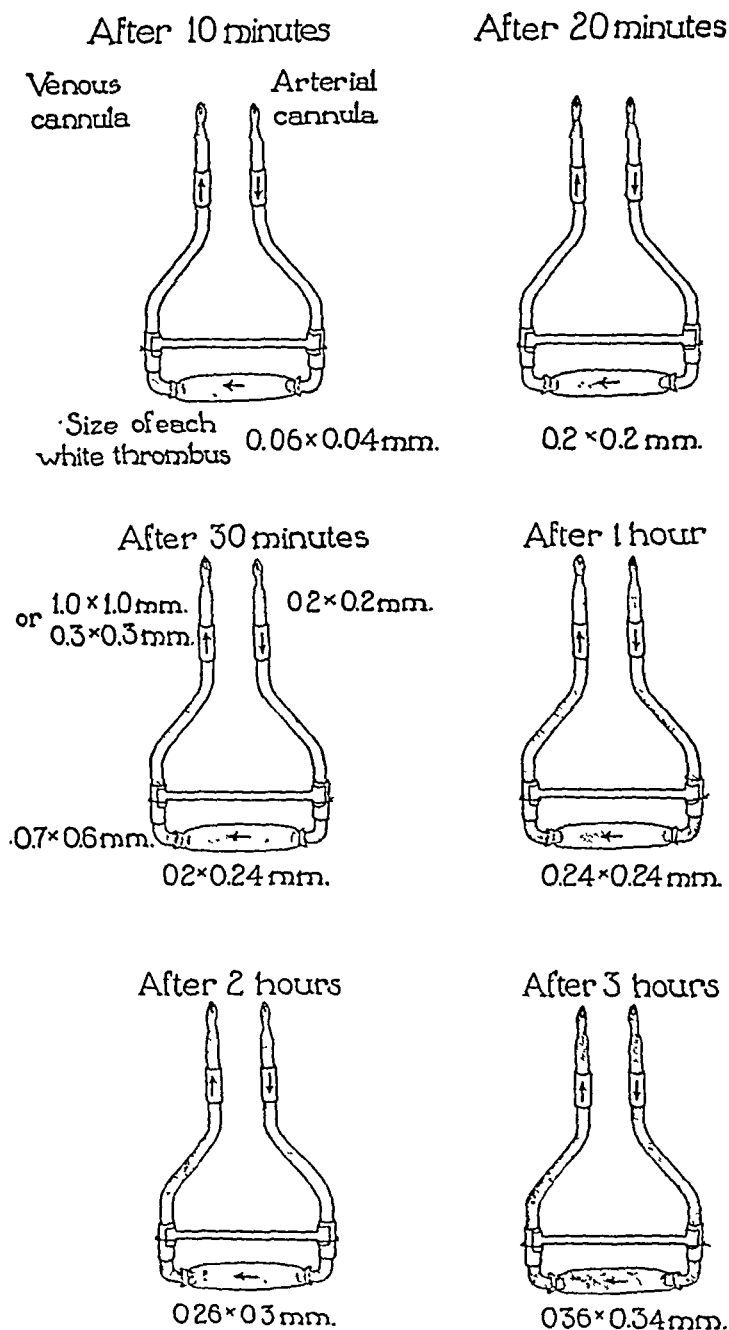
PLATES 2 AND 3.

(Received for publication, April 6, 1927.)

Welch (9) describes the steps in the formation of a thrombus after injury to the vessel wall, as follows: There is an accumulation of blood platelets adhering to the wall at the point of injury; leucocytes, which may at first be present in small numbers, increase rapidly, collecting at the margins of the platelet masses and between them. Not until the leucocytes have accumulated in considerable numbers, does the fibrin appear. Therefore, the formation of fibrin is secondary. Nevertheless, the fibrin formation plays a great part in the growth of red thrombi and the occurrence of obstruction and of intravascular clotting.

The effects of anticoagulants on the process of thrombosis with the new method of investigation of extracorporeal thrombosis was studied. Interest centers especially on the study of the mechanics of thrombosis and the preventive or retarding influence of anticoagulants which prohibit clotting for long periods. Here heparin, an antiproteolytic introduced by Howell (3), has been employed mainly. Howell (4) states that heparin is the anticoagulant always present in normal blood, and that it may be used intravascularly without injurious effects. Reed (7) found that repeated injections of this material always result in an increase of clotting time. In my experiments the heparin used (Hynson, Westcott and Dunning) was

\* Fellow of the Rockefeller Foundation.



TEXT-FIG. 1. Progression in formation of white thrombi.

standardized, 1 mg.<sup>1</sup> rendering 5 cc. of cat blood incoagulable *in vitro* for 24 hours. It is dissolved in physiologic sodium chloride solution, 20 mg. to 1 cc., and is injected intravenously in doses of 20 to 25 mg. for each kilo of body weight.

### *Experimental Studies with Heparin.*

Rabbits weighing about 2 kilos were anesthetized, and the carotid artery and jugular vein of the same or opposite sides cautiously exposed for more than 1 cm. All small vessels needing to be cut were first ligated to prevent subsequent loss of the blood rendered incoagulable by heparin injection. Then the anticoagulant was injected and the apparatus for extracorporeal thrombosis connected with the vessels of the animal. Since physiologic sodium chloride solution is usually regarded as an indifferent medium so far as blood is concerned, it was used in the container in which the collodion tube was immersed, as a control or standard experiment. The clotting time of the blood from the ear was tested often during the experiment by Mills' method; immediately after injection and persisting for about 4 hours the clotting time may be 30 minutes, or perhaps several hours. 4 and sometimes 5 hours after the injection of this amount of heparin the clotting time of the blood became shorter than 30 minutes, and tended to decrease rather quickly: for instance, in the next half hour, it became normal or sometimes less than normal.

There was considerable uniformity in the formation of white thrombi. From the results of thirty experiments, the sequence of events (Text-fig. 1) was as follows: After 5 minutes, no white thrombi were visible macroscopically, but a few small white thrombi were seen microscopically, which were chiefly composed of clumps of platelets, situated along the wrinkles of the bottom of the collodion tube. In size these clumps measured 0.06 by 0.04 or 0.04 by 0.04 mm. On and around the platelet clumps a few leucocytes were recognized. After 10 minutes numerous tiny white thrombi were seen macroscopically in the venous corner tube, or on one of the curved surfaces. In the collodion tube the number of white thrombi appeared increased, but they were still recognized only under the microscope. Also a few tiny ones were found in the venous cannula. After 15 or 20 minutes a few tiny white thrombi appeared in the arterial corner tube, while those in the venous cannula and corner

<sup>1</sup> 1 mg. of pure heparin renders 100 cc. of human blood incoagulable for 24 hours or more (Howell's personal communication).

tube increased in size and number. After 25 or 30 minutes numerous small white thrombi became macroscopically visible in the arterial cannula and in the curves of the side tubes, but were fewer on the arterial side. Those in the other parts increased in number and size. Sometimes the whole inner surface of the venous corner tube was coated with a number of white thrombi which were connected by threads of fibrin. The thrombi in the venous cannula and corner tube may be large (0.7 or 0.6 mm. in diameter); those in the cannula almost obstructed the constricted part. The blood stream in the apparatus gradually became slower, the laying down of the platelets was relatively decreased, and so the further growth of the white thrombi was relatively decreased. After 2 hours the thrombi had increased in size and number and the narrow part of the venous cannula was almost obstructed. In the collodion tube, many large white thrombi were obtainable (Fig. 1), often in the form of fern-like radiations, in the venous half or arterial half or sometimes in the center of the bottom of the collodion tube. These were propagated against the current. The site and growth of the white thrombi in the apparatus are shown in Text-fig. 1. The following protocol is typical of what was generally observed:

At 11.10 a.m. Rabbit 10, weighing 1400 gm., was injected intravenously with 40 mg. of heparin. At 12.00 the apparatus was connected and the collodion tube surrounded with physiologic sodium chloride solution of body temperature. The clotting time *in vitro* was more than 30 minutes. At 2.00 the apparatus was disconnected and the collodion tube detached. The blood remained totally incoagulable. All the surfaces of the apparatus were gently washed with physiologic sodium chloride solution. In the tubes of the venous side, numerous white thrombi were seen with a smaller number in the arterial tubes. The venous cannula seemed to be almost obstructed by a mass of white thrombi. In the collodion tube many white thrombi were observed in a fern-like radiating mass, which seen microscopically in side view appeared like a succession of hills and valleys. The size of the individual thrombus varies. Thus of thirteen thrombi seen under low power in one microscopic field, one thrombus measured 0.20 by 0.20 mm., four measured 0.16 by 0.12 mm., and eight 0.06 by 0.04 mm.

#### *Experiments with Hirudin.*

When hirudin was used instead of heparin, in a dosage of 10 mg. for each kilo of body weight in three experiments, the results were almost the same as when heparin was used (Fig. 2).

*The Mechanism of Thrombosis and the Influence of Heparin.*

These findings in the tubes should be discussed in connection with the mechanics of the blood flow. The blood in the carotid artery carries many platelets and leucocytes, which are hurled against the narrow part of the glass cannula. Platelets and leucocytes may adhere to the wall, but as the current is rapid, they usually become dislodged. The narrow part of the arterial corner tube is, as a rule, less often obstructed than that of the venous cannula, and then only at a later period. Wherever whirlpools, eddies and stagnation occur, platelets come in contact with foreign surfaces and they are agglutinated and white thrombi tend to form. At the spots where eddies form, relatively stagnant areas of blood are found; these favor agglutination probably through surface changes in the platelets.

The investigations show that in every part of the apparatus, even on the paraffined surface, the early stages of thrombosis can be demonstrated; though they appear earlier and are more marked, on the rough, irregular surface, for example, on the wrinkles of the collodion membrane. In the widest part of the apparatus, eddies occur on the surface in contact with the collodion and the platelets settle down.

The fact that clumps often assume a radiate or fern-like form in the lower part of the venous half of the collodion tube where whirlpools are seen through the collodion membrane, supports the von Recklinghausen theory (6). Eberth and Schimmelbusch's view (2, 8) in this regard commands attention but does not sufficiently explain the formation of platelet thrombi. Von Recklinghausen's theory appears more acceptable in explaining the mechanics of thrombosis. But in these experiments in the ampulla-like widening of the vessel, the thrombus forms mainly in the distal part of it and rarely in the proximal part. Platelets accumulate more in the distal part of the collodion tube than in the proximal end of it. Therefore, the explanation of the formation of the platelet clumps is to be found in the movement of the blood stream, rich in platelets and adequately slow, washing against the pathologic inner wall of the vessels, stagnating there and breaking into eddy currents against the irregular rough surface. If the stream is much slower, the white thrombi are chiefly formed in the arterial part of the apparatus; for instance in the collodion

tube it takes place in the arterial half of the lower part near the mouth of the corner tube. This is exactly in accord with the results of the experiments of Aschoff (1) and his coworkers with sand, showing that if the sand stream from the smaller vessel flows very slowly, a sand bank is formed in the bottom of its mouth.

*The Use of Anticoagulant as an Antithrombotic Substance.*

As Mason (5) has already stated, heparin might be effective against certain kinds of thrombosis, considering the thrombosis as a kind of intravascular clotting of the blood. But, since the first stage of autochthonous thrombosis is the deposition of platelets and not the deposition of fibrin, it does not suffice. At least single doses of the heparin used (Hynson, Westcott and Dunning) did not prevent the formation of white thrombi on foreign surfaces. In spite of the administration of a sufficient dose of this anticoagulant, and in spite of the incoagulability of the blood *in vitro*, white thrombi appear at the site where the conditions were conducive to clotting. The white thrombi gradually grew and sooner or later obstructed the lumen of the apparatus, and still later the lumen of the vessel. Therefore it appears that anticoagulants such as heparin<sup>2</sup> and hirudin in their form and in single doses do not afford complete protection against thrombosis. Experiments involving the continuous administration of heparin are contemplated.

In order to examine the structure of thrombi formed during the period of effective action of the anticoagulant, the apparatus itself may be removed and the ligatures holding the collodion tube in place may be cut at any desired time during the experiment. Thus the collodion tube with its contents is removed, its upper wall is carefully slit with a pair of delicate scissors and its inner surface cautiously washed with physiologic sodium chloride solution. Like a glass slide the collodion membrane is translucent enough to be examined directly under the microscope.

It is not desirable, perhaps, to dwell further on the character of the structure of the thrombus because that would but repeat Welch's

<sup>2</sup> The heparin used was supplied by Hynson, Westcott and Dunning. Professor Howell believes that pure heparin may entirely prevent the formation of white thrombi.

description (Figs. 3 and 4). Suffice it to say that his findings as to the structure of the white thrombi were verified by the method used in these experiments as well in serial sections as in the fresh state. But there is one respect in which my results appear to differ from his: the formation of fibrin is postponed on account of the anticoagulant action.

#### CONCLUSIONS.

1. Effects of anticoagulants, heparin and hirudin, on extracorporeal thrombosis were studied in thirty-three experiments by means of the extracorporeal vascular loop. In spite of adequate single doses of the anticoagulants white thrombi are formed and obstruction to flow may follow in the course of time, but the formation of red thrombi is markedly retarded.

2. The new method throws some light on the mechanics of thrombosis. The influences of foreign surfaces, of irregularities on the surface of the vessels, of whirlpools and of eddying motion of the blood current and of slowing and of stagnation with consequent prolonged contact with foreign surfaces are demonstrated, and these are analyzed in respect to the laying down of platelets and leucocytes.

3. The structure of white thrombi formed after anticoagulant injection is almost identical with that described by Welch. In one respect, however, the present results appear to differ from his: the formation of fibrin is postponed and retarded, and hence the collections of platelets are deposited in greater amount.

#### BIBLIOGRAPHY.

1. Aschoff, L., Lectures on pathology, New York, 1924, 253-278.
2. Eberth, J. C., and Schimmelbusch, C., Experimentelle Untersuchungen über Thrombose, *Arch. path. Anat. u. Physiol.*, 1886, ciii, 39.
3. Howell, W. H., and Holt, E., Two new factors in blood coagulation: heparin and pro-antithrombin, *Am. J. Physiol.*, 1918-19, xlvii, 328.
4. Howell, W. H., The purification of heparin and its presence in blood, *Am. J. Physiol.*, 1924-25, lxxi, 553.
5. Mason, E. C., A note on the use of heparin in blood transfusion, *J. Lab. and Clin. Med.*, 1924-25, x, 203.
6. von Recklinghausen, F. D., Handbuch der allgemeinen Pathologie des Kreislaufs und der Ernährung, Stuttgart, 1883.



7. Reed, C. I., The intravascular use of heparin, *Am. J. Physiol.*, 1925, lxxiv, 79.
8. Schimmelbusch, C., Die Blutplättchen und die Blutgerinnung, *Arch. path. Anat. u. Physiol.*, 1885, ci, 201.
9. Welch, W. H., The structure of white thrombi, Papers and addresses, Baltimore, 1920, i, 47.

## EXPLANATION OF PLATES.

## PLATE 2.

FIG. 1. Growths of white thrombi after heparin injection. *a*, after 10 minutes white thrombi appeared as a white line on the wrinkle of the center of the collodion tube. *b*, after 30 minutes the white thrombi grow up more remarkably. *c*, after 2 hours white thrombi multiply and are laid down in radiating form. *d*, after 3 hours.

FIG. 2. White thrombi after hirudin injection. *a*, after 2 hours. *b*, after 1 hour.

## PLATE 3.

FIG. 3. Hillocks of platelet thrombi 1 hour after anticoagulant injection. *a*, clumps of platelets. *b*, fibrin with white cells.

FIG. 4. Hillocks of platelet thrombi 3 hours after anticoagulant injection. *a*, clumps of platelets. *b*, fibrin with white cells.



FIG. 1.



FIG. 2.





FIG. 3.

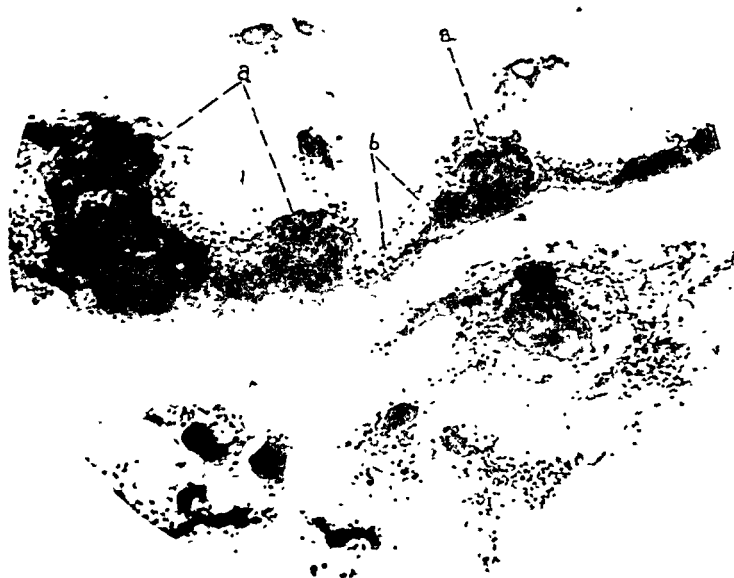


FIG. 4

(Shonoza: Extracorporeal thrombosis, III.)



## DIET AND TISSUE GROWTH.

### V. THE EFFECT OF DIETARY PROTEIN ON THE REMAINING KIDNEY OF ADULT WHITE RATS FOLLOWING A UNILATERAL NEPHRECTOMY.

BY THEODORE S. MOISE, M.D., AND ARTHUR H. SMITH, Ph.D.

(From the Department of Surgery and the Laboratory of Physiological Chemistry, Yale University, New Haven.)

PLATES 4 AND 5.

(Received for publication, April 14, 1927.)

#### INTRODUCTION.

The recent literature contains an increasing number of accounts of investigations on the effect of the ingestion of excessive amounts of protein on the kidneys of experimental animals. There is general agreement on the observation of a physiologic enlargement but conflicting results are reported concerning the part played by protein in the production of pathologic renal lesions.

The character of the physiologic enlargement has been the subject of prolonged dispute. The literature on this question has been summarized by Arataki (1926, *a*) who reported from his own studies on rats that there is no increase in the number of glomerular unit systems but that the enlargement consists in an increase in the size of both the glomeruli and tubules, an hypertrophy, together with an actual cellular increase of the interstitial tissue, an hyperplasia. He believes in addition that there is an hyperplasia of the constituent cells of the glomeruli and tubules. Furthermore he observed that the enlargement is incomplete after 80 days, and stated that in some instances it may approximate 100 per cent.

Although there is no increase in the number of glomeruli in the rat, in response to feeding excessive amounts of protein, the formation of these structures continues throughout the first 100 days of life according to Arataki (1926, *b*). He stated further that the total number of glomeruli at birth is about 10,000, and at the age of 25 days about 25,000, and that by the 100th day the maximum of 30,000 has been attained. This level is maintained to the age of 350 days but by the 500th day it has fallen to 22,000.

In a study of the compensatory enlargement of the opposite kidney after ligation of one ureter in white rats Hinman (1923) has stated that the histologic

changes of compensatory renal hypertrophy may be divided into three stages: first, initial congestion; second, active mitosis and growth, and third, the final equilibrium of hypertrophy. He mentioned further that the increase in the size of the intact kidney is complete after an average of 20 to 30 days; the average total increase is 20 per cent of the normal and doubling of the renal tissue is never observed. The question of diet is not discussed in either of the above papers.

Smith and Moise (1927) have reported an extensive series of experiments on the relation of the protein constituent of the diet to the rate and degree of compensatory enlargement of the remaining kidney after unilateral nephrectomy in adult white rats. These authors have observed that on the "standard" diet containing a moderate concentration of protein (18 per cent, Table I) there is a rapid increase (24 per cent) in the compensatory enlargement of the remaining kidney within the

TABLE I.  
*Composition of Experimental Diets.*

	"Standard food"		"High protein" food	
	Part of diet	Part of total calories	Part of diet	Part of total calories
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Protein (casein) <sup>1</sup> .....	18	14	85	76
Carbohydrate (raw cornstarch).....	51	39		
Fat {lard.....	22	47	7	24
{cod liver oil.....	5		4	
Salt mixture <sup>2</sup> .....	4	4	4	

700 mg. dried yeast daily given with each of the diets

<sup>1</sup> A commercial product containing 13 per cent nitrogen.

<sup>2</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

first 3 weeks. A similar series on a high protein diet (85 per cent casein) showed an extremely rapid gain to 63 per cent in 21 days. The curves of enlargement on the two diets continue approximately parallel and at 150 days the average enlargements are 48 and 121 per cent for the standard and high protein diets respectively. When adult white rats were fed diets containing increasing concentrations of protein, observations at a constant time interval (21 days) after unilateral nephrectomy showed that the increase in the size of the remaining kidney is directly proportional to the protein content of the food. The values vary from 24 per cent with the standard ration to 77 per cent with the high protein diet. All of the diets used are adequate for maintenance and growth. The large proportion of calories obtained from protein in some of the rations is the variable factor.

The material obtained from this investigation (Smith and Moise, 1927) affords a unique opportunity of studying the histologic changes in a large series of kidneys not only performing approximately double functional duty owing to the previous unilateral nephrectomy but also subjected to the additional burden of eliminating the metabolites of an extremely high protein diet (Table I). The purpose of the present communication is to report such structural changes and if possible to explain the discrepancy in the reports of other investigators.

In earlier studies of a somewhat similar nature (Newburgh, 1919), egg white, casein and soy beans were fed to three different groups of rabbits for periods of time varying from a few days to 7 months. Examination of the kidneys revealed evidence of acute and chronic renal disease, consisting chiefly of casts in tubules, varying degrees of injury to tubular epithelium and scarring of the cortex. There were no marked glomerular lesions. Newburgh and Clarkson (1923) observed marked dilatation of the tubules of the kidneys of rabbits fed diets containing lean beef amounting to 27 and 37 per cent of the ration for several months. There were no marked glomerular lesions. They called attention to the difference between the focal cortical scars occurring spontaneously in rabbits and the lesions observed in their experiments.

Evans and Risley (1925) described definite tubular and glomerular lesions in white rats fed a variety of substances containing between 27 and 75 per cent protein for periods from 6 to 15 months but the diets used in their studies were admittedly inadequate in salts and vitamins.

In all of the above mentioned experiments the diets were not only extreme but also inadequate and furthermore the early studies on rabbits (Newburgh, 1919) have been criticised because of the common occurrence of spontaneous lesions in this animal. Polvogt, McCollum and Simmonds (1923) made similar studies on white rats, with diets adequate for maintenance and growth and containing between 31 and 41 per cent protein. The time of feeding varied from a minimum of 129 to a maximum of 485 days. Congestion, degeneration of the tubular epithelium, casts and cellular detritus in the lumina of the tubules, hyaline material within the capsular space and adhesions between the glomerular tuft and Bowman's capsule were described in the kidneys of these animals.

Osborne, Mendel, Park and Winternitz (1927) observed tubular and glomerular lesions in the kidneys of eighteen white rats that had been fed high protein diets for long periods of time. These authors state that the lesions were focal in nature and were exceedingly slight in the entire group with the exception of seven animals, five of which were on a 40 per cent casein diet for 400 days or more. Exactly similar lesions were observed in five of the control animals maintained on Sherman's stock diets for over 500 days. The series also included eighteen rats on high protein diets (60 to 95 per cent) for periods varying from 200 to 360 days in which no renal lesions were observed. The average age of the animals on the high protein diet showing lesions was 348 days while those showing the lesions described as "severe" were over 400 days old.



On the other hand numerous investigators (Osborne, Mendel, Park and Darrow (1923), Osborne, Mendel, Park and Winternitz in a preliminary report (1925), Drummond, Crowden and Hill (1922), Reader and Drummond (1925), Miller (1925), Jackson and Riggs (1926), Anderson (1926), Addis, MacKay and MacKay (1926) and Kennedy (1926)) have reported that no pathologic structural changes were observed in the kidneys of intact animals (white rats, rabbits and cats) maintained on high protein diets for relatively prolonged periods. There is thus confusion in the literature on a point which is not only of academic interest but also of clinical importance.

#### EXPERIMENTAL.

Immediately following a right nephrectomy the animal was placed on the diet to be used and after a definite time interval the remaining kidney was removed for study. All of the kidneys were accurately weighed for determination of gross enlargement. Some were used for the determination of total solids while for the present study a large number were fixed in 10 per cent formalin, sectioned and stained with hemotoxylin and eosin.

The findings are based on the study of histologic sections from over 200 adult rats; namely, the left kidney from 92 animals on the "high protein" diet (85 per cent casein) and from a slightly smaller number of animals on the standard diet (18 per cent casein), for periods varying from 3 to 150 days after the control nephrectomy. The kidneys from another series of 60 animals on six diets varying in protein content from 30 per cent to 90 per cent casein for a period of 21 days following a right nephrectomy were also sectioned for histologic study. The animals were all somewhat under a year old (approximately 275 to 350 days) at the end of the experimental period.

In addition to the series of animals on the 18 per cent casein ration, the kidneys from groups of rats maintained on Sherman's diets "A" and "B" for periods between 350 and 565 days were examined.\* As further controls the removed right kidney from many of the experimental animals was available.

Although there is no significant anatomic evidence of renal injury in the animals on the standard diet, the kidneys of the rats on the "high protein" diet show some interesting structural changes. In the early and middle periods, excluding the phenomenon of gross enlargement, the observed changes are relatively inconsequential. However, in the late periods, namely, after 90, 120 and 150 days the kidneys of the animals on the high protein ration show significant glomerular and tubular changes (Table II).

\* The authors are greatly indebted to Professor E. A. Park of the Department of Pediatrics, Yale University, and Professor H. C. Sherman, Columbia University, for the material included in this group.

These changes represent actual lesions of the kidney and were observed in all animals on the high protein ration for 90 days or more. The lesions were not merely isolated focal changes but were conspicu-

TABLE II.

*Data on Rats Fed High Protein Diet and Showing Renal Lesions.*

Rat No	Age completion experiment	Initial body weight	Final body weight	Right kidney weight	Left kidney weight	Remarks
Interval after nephrectomy—90 days						The kidneys of the animals listed in this table (all rats maintained for 90 days or longer on the 85 per cent protein diet with the exception of those utilized for the total solid determinations) show the renal changes described above  These lesions become progressively more numerous and more widespread in the 90, 120 and 150 day periods  The second group in the 150 day interval (age 271 days) showed less enlargement and slightly less evidence of renal damage than was observed in the first group
594 <sup>1</sup>	292	310	320	1 225	3 205	
597	292	292	262	1 202	2 517	
602	295	279	268	1 239	2 321	
603	295	298	300	1 173	2 774	
606	295	309	300	1 250	2 865	
608	295	300	293	1 196	2 538	
Average <sup>2</sup> ...	288	271	1 171	2 389	Enlargement 113 per cent	
Interval after nephrectomy—120 days						
562	317	280	288	1 120	2 558	
580	317	282	262	1 132	2 244	
584	317	272	241	1 108	3 504	
590	322	293	285	1 287	2 529	
592	322	293	251	1 384	2 266	
Average <sup>2</sup> ...	288	276	1 205	2 608	Enlargement 123 per cent	
Interval after nephrectomy—150 days						
564	346	293	290	1 086	2 429	
568	346	299	284	1 121	2 381	
569	346	266	262	0 978	2 097	
575	348	291	271	1 190	2 400	
576	348	289	290	1 111	2 435	
578 <sup>1</sup>	348	284	214	1 106	1 912	
Average <sup>2</sup> ...	287	277	1 126	2 419	Enlargement 121 per cent	

TABLE II—Continued.

Rat No.	Age completion experiment	Initial body weight	Final body weight	Right kidney weight	Left kidney weight	24 hr. urine protein 120-140 days	Casts
Interval after nephrectomy—150 days							
667	271	245	184	1.147	1.718	119	Present
668	271	263	285	1.184	2.185	49	"
669	271	270	282	1.406	2.276	45	"
670	271	277	320	1.130	2.359	63	"
671	271	273	280	1.145	2.471	18	Absent
672	271	282	318	1.144	2.448	45	Present
673	271	261	238	1.012	1.928	70	"
674	271	257	315	1.176	2.541	84	"
675	271	234	291	0.981	1.968	60	"
676	271	243	271	1.008	1.827	40	"
Average...		261	278	1.133	2.172 Enlargement 79 per cent		

<sup>1</sup> Eliminated from computations of enlargement on account of large abscess (Rat 594) and marked emaciation (Rat 578).

<sup>2</sup> The average values for body weight and kidney weight are based on data from rats used for computation of total solids in addition to those listed in this table. For method of calculating per cent of renal enlargement see Smith, A. H., and Moise, T. S., *J. Exp. Med.*, 1927, xlv, 263.

ous and relatively widespread becoming progressively more marked in the 90, 120 and 150 day periods. The structures included in an injured area are frequently radial in form suggesting the involvement of individual glomerular unit systems.

The changes in the glomeruli consist in serum in the capsular spaces, proliferation of the epithelium of Bowman's capsule with and without adhesions between the tuft and capsule, fibrous thickening of Bowman's capsule, partial fibrosis of the glomerular tuft and in many instances infiltration of round cells in and around these areas (Figs. 1 to 4).

In the same periods, 90, 120 and 150 days, the tubules show a rather conspicuous desquamation of the lining epithelium with many fairly well preserved epithelial cells and amorphous material within the lumina.

There is a slight general tubular enlargement with a somewhat diffuse patchy distribution of areas of marked tubular dilatation. In these dilated tubules the epithelium may form a single relatively flat layer or may appear in many layers as projections into the tubules and in some instances practically fills the lumina. In many such tubules the lining epithelium consists of recently formed cells in which mitotic figures are seen as evidence of active epithelial proliferation. There are many focal groups of dilated tubules with absent or markedly flattened epithelial lining cells showing no evidence of cellular activity. In some dilated tubules hyaline casts are seen. There are other areas showing an increase in the interstitial tissue with a variable amount of round cell infiltration. There are no red blood cells in the tubules or the capsule spaces (Figs. 5 and 6).

These changes are chiefly late manifestations of the injury or irritation to which the kidney has been subjected. It seems probable that the irritation produces slight changes in the early periods but that the methods of study only demonstrate the later aspects of the process. The findings in a single animal on a 75 per cent casein diet for 21 days after a right nephrectomy suggest the correctness of this hypothesis. In the sections of the left kidney from this animal showing an enlargement of 96 per cent a few glomeruli are seen in which the tufts are large with widely separated cells. In some instances the tuft and capsule are in actual apposition or the space is bridged by strands of fibrin. These findings suggest fresh adhesions and are possible precursors of the fibrous adhesions observed in the late periods. Such apparently fresh adhesions are also seen in the sections of the kidneys 150 days after nephrectomy.

In addition to these actual lesions of the kidney there are other changes of a physiologic nature. In the normal rat's kidney a few glomeruli are seen in which the tubular epithelium extends into and partially lines the glomerular sac. This condition is observed more frequently in the high protein animals and may involve fully one-half of the circumference of Bowman's capsule. It seems probable that this is a compensatory phenomenon whereby the tubular epithelium, in response to a functional demand, partially replaces the normal lining of Bowman's capsule.

In the early periods in spite of the extraordinarily rapid renal en-

thirty-four determinations was 49 mg., a value which corresponds fairly well with the data on the younger rats.

Samples of urine for casts were obtained directly from the urethral orifice on a slide by causing the rat to breathe ether vapor.

In Periods III and IV, those intervals when structural damage was observed, 112 samples from 13 rats on the protein-rich diet, casts were observed in 33 cases on 12 animals while in 107 samples from 11 rats on the control food, only 3 instances of casts were seen in 2 animals. Three observations of casts were made in the former group of rats in Periods I and II while none were found in the latter group. The increased excretion of protein in the urine and the greater incidence of casts furnish striking corroborative evidence for renal injury in the remaining kidney of the animals on the high protein diet.

#### DISCUSSION.

The necessity for adequate control in the differentiation of spontaneous renal disease and changes attributed to any experimental procedure is quite obvious and accordingly it is advisable to mention the occurrence of spontaneous renal lesions in rats. In a study of wild rats, Ophüls and McCoy (1912) have noted the common occurrence of a characteristic type of nephritis. Reader and Drummond (1925) observe that focal renal lesions are not uncommon in white rats. In the present investigation the occurrence of such changes in normal animals has been recognized.

There is little doubt that the structural changes observed in the animals on the high protein diet are a result of the experimental procedure and are not purely a part of the spontaneous renal changes that occur in white rats. In the present experiments actual renal lesions, including a proliferation of the tubular and capsular epithelium, adhesions between and a partial fibrosis of the glomerular tuft and capsule, were observed in animals maintained for 90 days or more after a unilateral nephrectomy on a diet containing 85 per cent casein. The kidneys of the animals on the high protein ration also showed marked tubular dilatation, including a rather diffuse change with evidence of active regeneration of the epithelium as well as other areas apparently atrophic in nature, showing groups of dilated tubules with a flattening or absence of the epithelium and without evidence of regeneration.

These latter groups of dilated tubules may be secondary to an injured glomerulus.

The lesions observed in animals fed the high protein food are widespread in distribution in contrast to the relatively inconspicuous focal lesions observed in the control animals. Occasional scattered focal lesions are not infrequently observed in normal adult white rats more than a year old and accordingly in the interpretation of our data no significance has been attached to isolated focal areas, of tubular dilatation, and, less frequently, of glomerular adhesions and capsular thickening. They are apparently phenomena of senescence and were observed regardless of the composition of the diet and became progressively more common with age. In the kidneys of control animals maintained on a normal dietary régime for 120 and 271 days respectively no focal lesions were found. Observations were made on the kidneys of rats maintained on Sherman's diets "A" and "B" for 350, 361, 372, 414 and between 500 and 565 days. At 350 days these focal lesions were inconspicuous or entirely absent but became progressively more common with successive periods. After 500 days they were present in a high percentage of animals. It is interesting to note in this connection that Arataki has observed a normal decrease in the total number of glomeruli with advancing age and it is possible that the spontaneous focal lesions are secondary to the normal process of glomerular involution.

In the experimental animals maintained on the standard ration such changes were practically never seen while in the animals on the high protein régime the focal lesions were larger and more frequently observed than in normal rats.

In searching for an explanation of the disagreement between the results reported by other investigators the important factors, namely, the animal, the diet and the duration of the experiment, must be considered. Although these points have been satisfactorily controlled in most of the investigations a careful analysis of the results suggests that a due consideration has not been given to the factor of age. The greater part of the reported experiments were initiated on young animals during the period in which active formation of glomeruli (Arataki, 1926, *b*) is taking place and it seems logical to assume that an animal may have greater powers of adaptation during this early period.

The fact that most of the observations mentioned in the literature reporting no pathologic renal changes were initiated on young rats, favors the above suggestion that such animals are less susceptible to the injurious effect of the ingestion of high concentration of protein. There are, however, experiments reporting negative results with a small number of adult animals (Miller, 1925, and Jackson and Riggs, 1926). In general the positive reports of the production of renal lesions by feeding of excessive amounts of protein have one or both of two serious objections, namely, the choice of the experimental animal or the inadequate and ill balanced diets in regard to the important accessory food requirements. Both of these factors are controlled in the experiments reported by Polvogt, McCollum and Simmonds (1923) who mention the production of lesions by feeding high protein diets. In their experiments young animals were placed on the diet in question for periods ranging from 129 to 485 days. An analysis of their protocols shows that the lesion described in the shorter periods consists in the variable factors of congestion with cellular detritus and serum in the tubules while the really significant lesions, the glomerular adhesions, are described only in animals that have been on the high protein diet for 400 days or more. Similarly, in the experiments reported by Osborne, Mendel, Park and Winternitz (1927), all animals having "severe" lesions were more than 400 days old. These findings are confirmatory evidence of the truth of our suggestion that the kidneys of young animals during the formative stage may be less susceptible to the irritating factor in high protein diets than adult animals.

Although the exact mechanism whereby structural lesions develop in animals ingesting excessive amounts of protein is not clear, two probable factors are suggested: either the changes are associated with the increased burden upon the kidney or, what is more probable, they result from the elimination of some injurious product of protein metabolism.

In the present studies such factors may include the high acidity of the urine, the phosphates, the urea and possibly amino acids. Addis, MacKay and MacKay (1926) have shown that no anatomic lesions are produced in a normal white rat with two kidneys by feeding diets containing an excessive acid or phosphate content. Neither Hinman (1923), Osborne, Mendel, Park and Winternitz (1927) nor MacKay,

MacKay and Addis (1927) were able to demonstrate consistent renal enlargement when quantities of urea equivalent to the protein in diet were given. These observations suggest that increased work is not the only important factor but they do not eliminate the possibility that the persistent effect of unusually large quantities of urea may result in structural damage to the kidney under the conditions of the present experiments. On the other hand Newburgh and Marsh *et al.* (1925) have described renal lesions after the intravenous administration of certain amino acids (lysine, histidine, tyrosine, tryptophane and cystine). The proliferative character of the epithelial changes suggests the persistent action of a mild irritant producing no extensive necrosis but by continual or frequently repeated slight injuries finally resulting in the lesions described above. Further investigation is necessary to determine the specific factor involved in the production of the lesions under the experimental conditions outlined in the present study.

#### SUMMARY.

The effects of the ingestion of diets containing different concentrations of protein on the remaining kidney in adult white rats after a unilateral nephrectomy has been studied.

In the animals on the high protein diet (85 per cent casein), actual glomerular and tubular lesions were observed in the kidneys of animals maintained for 90, 120 and 150 days after nephrectomy.

In the animals on the standard ration, 18 per cent casein, no significant renal lesions were observed within the experimental period.

Spontaneous focal lesions in the kidneys of rats maintained on Sherman's diets "A" and "B" were inconspicuous at the age of 350 days but became progressively more frequent and were commonly observed after 500 days. The animals on the high protein and standard rations were all under 350 days old at the completion of the experiment.

It is suggested that the age factor is of importance in that young animals may have greater powers of adaptation in withstanding the injurious effect of high protein rations.

The animals on the high protein ration excreted definitely larger quantities of protein in the urine, and showed a higher incidence of casts in periods roughly corresponding to those in which anatomic lesions were observed than did the rats on the standard diet.



## BIBLIOGRAPHY.

- Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.*, 1926, lxxi, 139.
- Anderson, H., *Arch. Int. Med.*, 1926, xxxvii, 313.
- Arataki, M., *Am. J. Anat.*, 1926, a, xxxvi, 437; 1926, b, xxxvi, 399.
- Drummond, J. C., Crowden, G. P., and Hill, E. L. G., *J. Physiol.*, 1922, lvi, 413.
- Evans, N., and Risley, E. H., *California and Western Med.*, 1925, xxiii, 437.
- Hinman, F., *J. Urol.*, 1923, ix, 289.
- Jackson, H., Jr., and Riggs, M. D., *J. Biol. Chem.*, 1926, lxxvii, 101.
- Kennedy, W. P., *Quart. J. Exp. Physiol.*, 1926, xvi, 281.
- MacKay, L. L., MacKay, E. M., and Addis, T., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 336.
- Miller, A. J., *J. Exp. Med.*, 1925, xlii, 897.
- Nelson, V. E., and McCay, C. M., *Proc. Iowa Acad. Sc.*, 1924, xxxi, 286.
- Newburgh, L. H., *Arch. Int. Med.*, 1919, xxiv, 359.
- Newburgh, L. H., and Clarkson, S., *Arch. Int. Med.*, 1923, xxxii, 850.
- Newburgh, L. H., Marsh, P. L., Clarkson, S., and Curtis, A. C., *J. Am. Med. Assn.*, 1925, lxxxv, 1703.
- Ophüls, W., and McCoy, G. W., *J. Med. Research*, 1912, xxvi, 249.
- Osborne, T. B., Mendel, L. B., Park, E. A., and Darrow, D., *Proc. Soc. Exp. Biol. and Med.*, 1923, xx, 452.
- Osborne, T. B., Mendel, L. B., Park, E. A., and Winternitz, M. C., *Am. J. Physiol.*, 1925, lxxii, 222.
- Osborne, T. B., Mendel, L. B., Park, E. A., and Winternitz, M. C., *J. Biol. Chem.*, 1927, lxxi, 317.
- Polvogt, L. M., McCollum, E. V., and Simmonds, N., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 168.
- Reader, V. B., and Drummond, J. C., *J. Physiol.*, 1925, lix, 472.
- Smith, A. H., and Moise, T. S., *J. Exp. Med.*, 1927, xlv, 263.

## EXPLANATION OF PLATES.

## PLATE 4.

FIG. 1. Rat 584. High protein diet for 120 days following a right nephrectomy. There is a small fibrous adhesion between the tuft and Bowman's capsule.  $\times$  about 305.

FIG. 2. Rat 564. High protein diet 150 days following a right nephrectomy. There is marked proliferation of the epithelium lining Bowman's capsule with small adhesions between the tuft and capsule. There is a focal accumulation of small round cells adjacent to the glomerulus.  $\times$  about 305.

FIG. 3. Rat 562. High protein diet for 120 days following a right nephrectomy. There is a large fibrous adhesion between the tuft and capsule involving approximately one-half of the circumference of the glomerulus.  $\times$  about 305.

## PLATE 5.

FIG. 4. Rat 674. High protein diet for 150 days following a right nephrectomy. There is a large fibrous adhesion between the tuft and capsule with fibrosis of approximately one-half of the tuft.  $\times$  about 300.

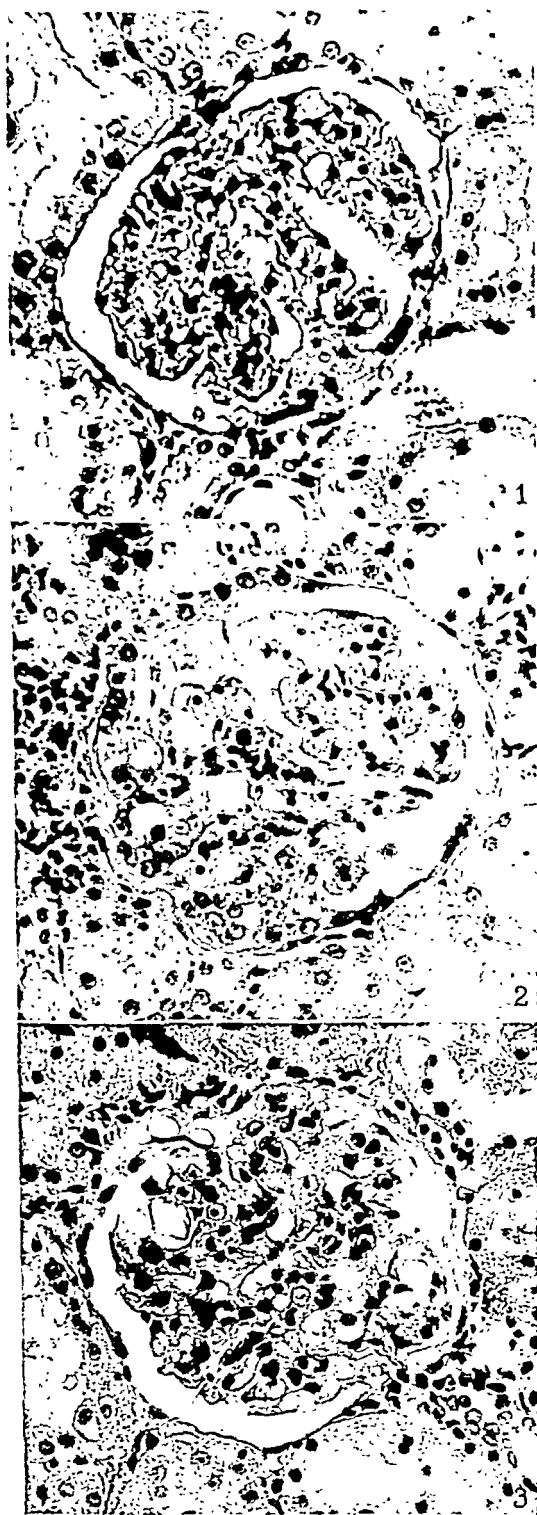
FIG. 5. Rate 564. High protein diet for 150 days following a right nephrectomy. The figure shows two dilated tubules lined with newly formed epithelium. A mitotic figure is seen in the center of the figure.  $\times$  about 360.

FIG. 6. *A*, Rat 667; *B*, Rat 674. High protein diet for 150 days following a right nephrectomy.

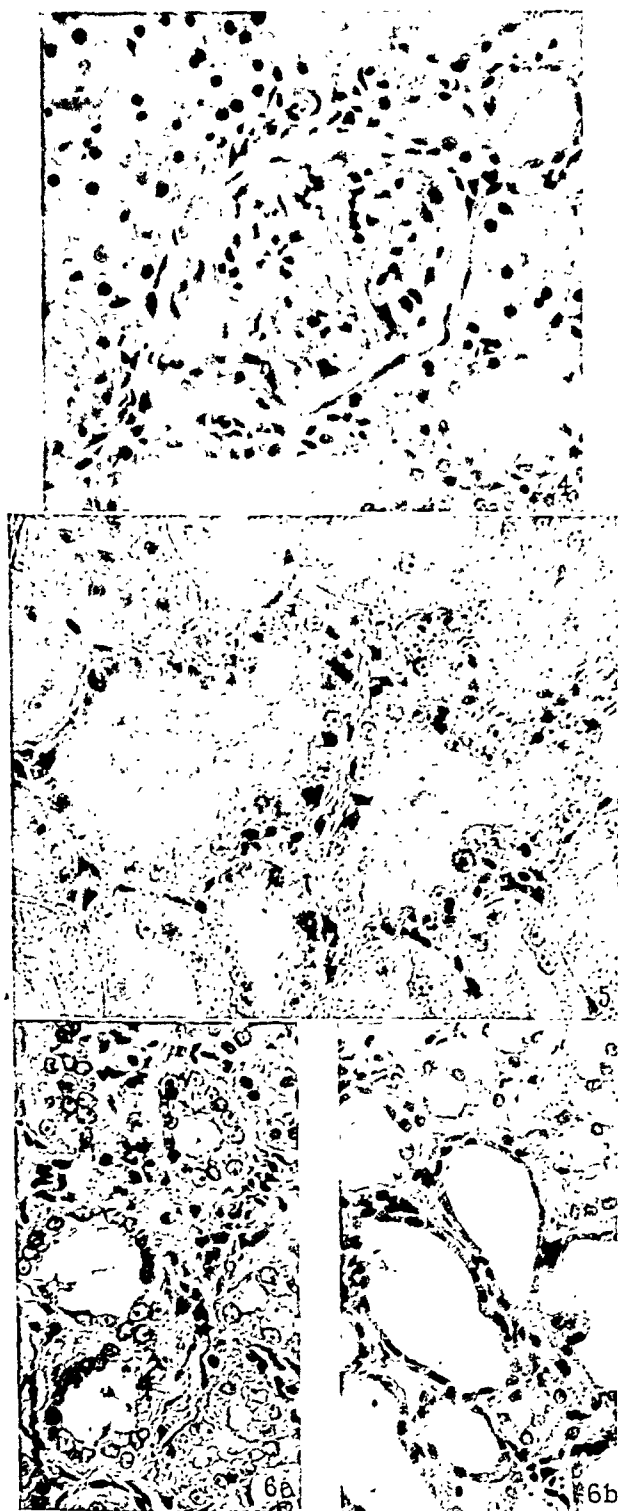
*A*—The tubules are moderately dilated. The lining epithelium is cubical with clear and vesicular nuclei. The tubules are widely separated due to an increase in the interstitial tissue.  $\times$  about 300.

*B*—The tubules are markedly dilated. The lining epithelium is flat. There is no evidence of cellular activity.  $\times$  about 300.











## CELL RESPIRATION STUDIES.

### I. A MICROSPIROMETER FOR THE CONTINUOUS STUDY OF THE OXYGEN ABSORPTION BY LIVING CELLS.\*

BY BLAKE C. WILBUR, M.D., GENEVA A. DALAND, AND JOHN COHEN, M.D.

(From the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University, Boston.)

(Received for publication, April 4, 1927.)

The study of the ultimate metabolism of living organisms resolves itself into the analysis of the process in individual cells. While certain facts may be learned from the study of unicellular organisms, the specialized cells of the more complicated structures of mammals present additional problems. For the study of the respiration of such cells, numerous methods have been devised. Some depend on the oxidation or reduction of chemical agents, and others depend on the principle of gas analysis. The criticisms of most of the published methods are that the cells are exposed to abnormal conditions of environment, temperature, and osmotic pressure, and that the quantitative methods do not permit the continuous study of the same cells over long periods of time. It is possible to note the disappearance of oxygen in whole blood by determining the oxygen content at the beginning and at the end of a definite period, but in the actual chemical analysis the cells are killed. In such methods the number of observations is limited and the composition and identity of the cells are naturally not homogeneous.

To obviate these criticisms, an apparatus was designed to allow quantitative measurements of the volume of oxygen over a sample of cells. In the experiments with this apparatus seeds, pieces of tissue, and blood corpuscles were used, without disturbing their vital processes. The living structures during the time of the experiment

\* The expenses of this investigation have been defrayed in part by a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.



were kept at a uniform temperature and in intimate relation with the respiratory medium.

The principle on which this microspirometer works is similar to that of most forms of closed space respiration apparatus. It has the advantage of this type of apparatus in that very accurate determination of the oxygen consumption can be obtained with comparative ease over short periods of time. It may be used for experiments involving a composition of gases differing from the atmosphere. The variations in the volume of the oxygen in the respiratory chamber may be measured during a given interval of time if all the carbon dioxide produced is absorbed by a solution of potassium hydroxide. The difference in the volume at the beginning and at the end of a given period, when the temperature and pressure are kept constant, is a measure of the amount of oxygen absorbed. Readings of the volume of oxygen used may be made at regular intervals during the course of the experiment.

Among the several forms of closed space microspirometers described by others, the differential microspirometer for the direct measurement of the consumption of oxygen designed by Barcroft and Roberts (1) and later modified by Krogh (2), and by Warburg (3), and also the one used by Winterstein (4), present certain technical problems, when blood is used, which make the results difficult to interpret. The apparatus designed by Koehler (5), though easy to manipulate, is not practical for small amounts of blood. Various forms of apparatus such as the Van Slyke (6) and the Haldane (7) measure changes in oxygen content, but the cells are killed in the process.

Such factors as the difference in vapor pressure between the material examined and the absorbing fluid, the difference in vapor tensions over the leveling fluids in the differential capillary tubes, the incomplete absorption of carbon dioxide, insufficient shaking or stirring, and the like, are sources of error which modify appreciably the results. The apparatus designed in this laboratory appears to eliminate these sources of error.

### *The Apparatus.*

A diagram of the microspirometer described below is shown in Fig. 1. It is constructed of Pyrex glass.

For convenience the whole apparatus is mounted on a board and placed on a stand so that it can be put in a water bath with the level of the water just above the transverse connections and stop-cocks.

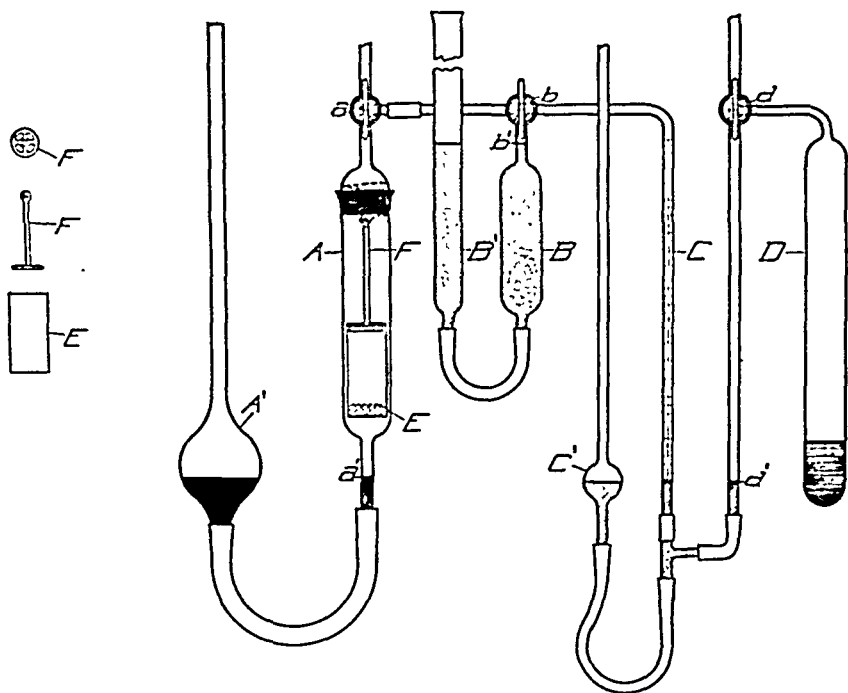


FIG. 1. *A*, metabolism chamber with reservoir *A'* for mercury. *B*, absorption chamber with reservoir *B'* for potassium hydroxide. *C*, manometer tube with reservoir *C'* for bile solution. *D*, compensation tube. *E*, cup to hold material for study. *F*, stirring rod. At the left the face view shows the perforated disk. *a*, *b*, and *d* represent the three-way stop-cocks which are attached to the chambers with corresponding letters. *a'*, *b'*, and *d'* represent the level to which the solutions are raised when readings are taken.

The microspirometer consists essentially of four parts:

1. The respiration or metabolism chamber *A* which holds a small cup *E* into which the material to be studied is placed.

The outside measurement of the metabolism chamber *A* is  $19 \times 3.9$  cm. The bottom narrows down leading through a capillary tube into a rubber hose connected with the mercury reservoir *A'*, the diameter of which is 7 cm. The top of

the metabolism chamber is closed with a tightly fitting ground glass stopper, on which there is a three-way stop-cock leading from this chamber to the outside and to the absorption chamber. Inside the stopper there may be placed a small brass spring to suspend the stirring rod *F*. The stirring rod consists of a perforated glass disk, 2.3 cm. in diameter, on the end of a glass rod 6 cm. long. The cup *E* in the metabolism chamber is 6.5 cm. deep and 3.3 cm. in diameter.

2. The absorption chamber *B* which contains the potassium hydroxide solution to absorb the carbon dioxide in the air driven over from the metabolism chamber.

The absorption chamber measures  $15 \times 3.5$  cm. Its outlet to the reservoir *B'* is 0.5 cm. in diameter and the reservoir *B'* is  $45 \times 25$  cm.

The concentration of the potassium hydroxide used is 0.96 N.

3. The manometer tube *C* which indicates the change of volume resulting from the consumption of oxygen by the material studied.

The manometer tube has a bore of about 0.3 cm. and it is 30 cm. long. It is graduated into tenths of a cm. and has a volume of 2.2 cc. Calibration shows each cm. to equal 0.0735 cc. at  $37.5^{\circ}\text{C}$ . The reservoir *C'* is 3.5 cm. in diameter.

A solution of bile salt containing 23 gm. sodium chloride and 5 gm. sodium taurocholate in 500 cc. of water (8) is used in the manometer. This solution has the advantage of dissolving fat, and therefore runs evenly in the capillary tubes. It does not absorb oxygen or carbon dioxide to a significant extent.

4. The compensating tube *D*, which is attached to the manometer to correct for changes in temperature and barometric pressure after the apparatus is closed.

This chamber has the same volume (148 cc.) as the metabolism chamber *A*. For each cc. of material added to the latter an equal volume of water should be placed in the compensating chamber *D*.

### *Procedure.*

The reservoirs attached to the apparatus are filled with the proper fluids. Enough mercury is placed in the reservoir *A'* attached to the metabolism chamber *A*, to raise the cup *E* to the top of the chamber when the reservoir is elevated. 2 or 3 drops of the bile solution are placed on top of the mercury in the metabolizing chamber *A* to prevent the mercury from adhering to the sides and thus interfering with the readings.

The potassium hydroxide solution is placed in the reservoir  $B'$  and allowed to fill the absorption chamber  $B$  to the mark  $b'$ . The manometer  $C$  and its reservoir  $C'$  are partly filled with the bile solution.

To make the apparatus absolutely air-tight the stop-cocks must be cleaned and lubricated with a suitable stop-cock grease (e.g. Ramsay) for each experiment.

When the apparatus is ready, the material to be studied is placed in the cup inside the metabolism chamber  $A$ . The ground glass stopper is well lubricated with stop-cock grease and sealed.

The entire apparatus is then placed in a water bath maintained at  $37.5^{\circ}\text{C}$ ., or any other desired temperature. In the water bath used it was found possible to maintain a temperature of  $37.5^{\circ}\text{C}$ . with a maximum variation of  $0.2^{\circ}$ . The temperature may be observed every 5 to 15 minutes on a Beckmann metastatic thermometer, during the course of each experiment (1 to 5 hours). The water bath is heated with electric light bulbs, controlled by a mercury level thermostat. The water is kept in circulation by a motor-driven stirring apparatus. An hour is allowed for the apparatus to acquire the temperature of the bath. The microspirometer is left in the water bath throughout the experiment and readings may be made through a glass window in the side of the water bath.

When a fluid substance, as blood, is to be tested it may be added, after the apparatus is warmed, through a long, hollow needle, inserted through the stop-cock  $a$ .

Before observations are commenced all of the stop-cocks are opened to the air, then the mercury is leveled to the mark  $a'$ , the potassium hydroxide is leveled to the mark  $b'$ , and the bile solution is brought to the mark  $d'$  of the compensating tube. The manometer reading is noted and then the apparatus is closed to the air with the fluids at these levels. Stop-cock  $b$  is then closed so that the absorption chamber connects only with the metabolism chamber, which is closed off from the outside by stop-cock  $a$ . The compensating tube is closed so that it connects only with the manometer tube.

When blood or a fluid is studied it should be stirred constantly. To do so the mercury reservoir is raised and lowered slowly but continuously, so that the perforated disk suspended in the metabolism chamber agitates the fluid as it rises and falls. If agitation is unneces-

sary, as with solid substances, then at any desired period of time the mercury reservoir is raised and lowered several times to drive the gases over into the absorption chamber.

To make a reading of the new volume, the mercury is lowered slowly until the potassium hydroxide rises to the mark  $b'$ . When the solution is at this level, stop-cock  $b$  is turned so that the absorption chamber is closed off and the metabolism chamber is connected with the manometer. The mercury is now set at the mark  $a'$ . To make the reading, the bile solution must be brought to the mark  $d'$  in the compensating tube, and then the level in the manometer recorded. Leveling the bile solution at the mark  $d'$  corrects any changes in volume due to barometric and temperature variations. The diminution of volume recorded after this procedure is the result of the consumption of oxygen by the material studied, as the carbon dioxide produced has been absorbed. Any carbon dioxide remaining in solution does not influence the results as it is oxygen consumption that is desired. Any oxygen that may be in solution in the material studied and in the potassium hydroxide remains practically constant throughout the experiment, because the change in the tension is too small to affect measurably the amount of the dissolved gas. If a reading is taken before absorption of the carbon dioxide the quantity produced may be determined by reading the volume before stirring. This will give the difference between the oxygen consumed and the carbon dioxide produced, and after the absorption of carbon dioxide the remaining volume indicates the amount of oxygen consumed.

The apparatus must be calibrated by liberating or withdrawing known volumes of gas and measuring the change in level of the bile column in the manometer tube. The gas may be liberated chemically, or air or carbon dioxide may be added from a graduated pipette or syringe through stop-cock  $a$ . In experiments of this type the observed readings were what they should have been and well within the limits of experimental error. In the microspirometer described above, the volume change in the manometer tube multiplied by 3.2 gives the volume change in the metabolism chamber.

The error in reading the level of the bile solution in the manometer tube is 0.5 mm., which is equivalent to 0.00367 cc. of gas in the manometer or 0.01174 cc. in the volume of the metabolism chamber. The

bore of the capillary tubes, in which the mercury of the metabolizing chamber at  $a'$  and the potassium hydroxide at  $b'$  are leveled, is practically the same as that of the manometer, so that the error of reading these levels is no greater. When blood is placed in the microspirometer and the stop-cocks closed to the outer air, the gas in the chamber slowly increases in volume, during the course of 5 to 30 minutes. A decrease due to oxygen absorption by the cells becomes apparent after the maximum increase has been attained and equilibrium has been reached. The preliminary increase in volume may be the result of several factors. The blood cools slightly when drawn and there is an increase in the temperature when it is put into the apparatus. This may result in changed vapor tension as well as changed solubility of dissolved gases. It is also possible that, under the conditions of the experiment, methemoglobin may be formed, with liberation of oxygen. Spectroscopic examination of the blood after the observations had been completed were made by Dr. Mirsky and Dr. Anson, but the amount of methemoglobin, if present, was too small to be noted by the spectroscope.

The procedure used for studying blood has been as follows:

8 cc. of human blood are withdrawn from the median vein of the arm into a syringe containing a solution of heparin<sup>1</sup> as an anticoagulant. The syringe is gently agitated to thoroughly mix the heparin with the blood. If it is desired, the blood may be saturated with oxygen. 5 cc. are then put into the cup  $E$  of the warmed microspirometer, through a hollow needle, inserted in stop-cock  $a$ . The fluids are brought to a level at their respective marks and the apparatus closed to the outside air. Stirring is commenced at once and continued throughout the experiment in order to keep the blood oxygenated and continually in motion. Readings are taken at about 10 minute intervals. The rest of the blood may be used for quantitative morphological studies.

When isolated corpuscles are used instead of whole blood, the procedure is the same. The blood is drawn into a syringe containing heparin and then centrifuged. The plasma and white cells are removed separately. Enough of the plasma is then added to the cells to make up the desired volume. Quantitative studies of the cells in the resulting suspension may be made.

<sup>1</sup> 0.05 cc. of a 2 per cent solution of heparin is used for 5 cc. of normal blood. Twice as much is used when working with leucemic blood, with a high white blood cell count.

One may readily observe the consumption of oxygen by many forms of living material. At the suggestion of Dr. Alfred C. Redfield, sprouting radish and lupin seeds were used for this purpose and the rate of oxygen consumption was measured at frequent intervals over a period of 2 days. The results show a uniform rate of oxygen consumption with the growth of the seeds.

The amount of oxygen used by normal or tumor tissues is readily measurable by this spirometer.

In working with an apparatus of this type it should be borne in mind that the actual rate is a function of the shape and size of the vessel in which the material is exposed and the rate at which it is stirred. If the process which liberates or consumes gas proceeds at a uniform rate, and the rate of stirring (and, therefore, the rate of exposure of carbon dioxide, when formed, to the potassium hydroxide) is uniform, the rate per hour is the same at different times. If, under these conditions, the rate per hour differs from time to time, it is suggestive that the process does not proceed at a uniform speed. Data as to rate are comparable only when the stirring is uniform. The "lag" of the mercury in the capillary tube acts as a governor, controlling the speed of the stirring, and this helps to keep it uniform.

Comparative studies of the oxygen consumption of normal and pathological bloods are of value if they are carried out under similar technical conditions.

#### CONCLUSION.

A description is given of a closed space respiration apparatus which can be used to determine the amount of gas used or liberated by living blood or tissue cells, or chemical substances. Continuous observations can be made and repeated measurements recorded without interrupting the vital processes or destroying the cells.

Studies of the oxygen consumption by whole blood in normal individuals and in patients with leucocytosis and myelogenous leucemia, as well as by white cells suspended in plasma, will be reported in subsequent papers.

It is a pleasure to acknowledge our indebtedness to Dr. George R. Minot, Dr. Thomas E. Buckman, Dr. Cyrus C. Sturgis, and Dr.

Raphael Isaacs for the valuable criticisms and suggestions which have made this work possible.

#### BIBLIOGRAPHY.

1. Barcroft, J., and Roberts, F. F., Improvements in the technique of blood-gas analysis, *J. Physiol.*, 1909-10, xxxix, 429.
2. Krogh, A., Ein Mikrorespirationsapparat und einige damit ausgeführte Versuche über die Temperatur-Stoffwechselkurve von Insektenpuppen, *Biochem. Z.*, 1914, lxii, 266.
3. Warburg, O., Versuche an überlebendem Carcinomgewebe *Biochem. Z.*, 1923, cxlii, 317.
4. Winterstein, H., Ein Apparat zur Mikroblutgasanalyse und Mikrorespirometrie, *Biochem. Z.*, 1912, xlv, 440.
5. Koehler, A. E., An apparatus for measuring the oxygen consumption of tissues, *J. Biol. Chem.*, 1925, lxiii, 475.
6. Van Slyke, D. D., Studies in acidosis. II, *J. Biol. Chem.*, 1917, xxx, 347.
7. Haldane, J. S., A new form of apparatus for measuring the respiratory exchange of animals, *J. Physiol.*, 1892, xiii, 419; A new apparatus for accurate blood-gas analysis, *J. Path. and Bact.*, 1919-20, xxiii, 443.
8. Brodie, T. G., Some new forms of apparatus for the analysis of the gases of the blood by the chemical method, *J. Physiol.*, 1909-10, xxxix, 391.





## CELL RESPIRATION STUDIES.

### II. A COMPARATIVE STUDY OF THE OXYGEN CONSUMPTION OF BLOOD FROM NORMAL INDIVIDUALS AND PATIENTS WITH INCREASED LEUCOCYTE COUNTS (SEPSIS; CHRONIC MYELOGENOUS LEUCEMIA).

By GENEVA A. DALAND AND RAPHAEL ISAACS, M.D.

(From the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University, Boston.)

(Received for publication, April 4, 1927.)

The observation by Warburg (1) that the amount of oxygen used by a slice of Flexner-Jobling rat carcinoma was less than that absorbed by such normal tissues as liver or kidney, has opened the way for the further study of the metabolism of tumor and other rapidly growing cells. The immature white blood cells of myelogenous leukemia resemble somewhat those of the malignant neoplasms in their growth rate and behavior, and response to exposures to Roentgen rays. They serve as excellent material for the study of oxygen consumption because of their availability and the ease with which they can be manipulated *in vitro*.

Patients with leukemia frequently have an elevated basal metabolic rate, particularly when the blood contains many immature cells (Riddle and Sturgis (2)). The possibility of a causal relationship between the rate of oxygen absorption by the patient and the degree of maturation of the blood cells presents a problem toward the solution of which this study of cell respiration in the microspirometer offers some data.

The studies reported here include observations on the blood of normal individuals, a patient with a leucocytosis from sepsis, and patients with chronic myelogenous leukemia in various stages of the disease.

*Methods.*

The details of the use of the special microspirometer have been reported elsewhere (3). The method of Lundsgaard and Möller (4) was used in the determination of the oxygen content of the blood in the Van Slyke blood gas apparatus. Blood from the arm veins was used and the enumeration of the cells was made by the standard methods. In making the differential leucocyte counts, 500 to 1000 white blood cells were studied.

## DATA.

*Normal Blood.*

When the venous blood from a normal individual is transferred directly to the microspirometer part of the oxygen is used to convert the reduced hemoglobin to oxyhemoglobin. The amount used in excess of this represents the oxygen used for metabolism by the cells.

The oxygen content of a specimen of blood can be measured at the beginning and end of an experiment and the amount of oxygen necessary for saturation of the hemoglobin can also be determined by use of the Van Slyke blood gas apparatus. The microspirometer, however, shows the total amount of oxygen used during a similar period of time.

The results are reported of sixteen observations in which the hemoglobin was unsaturated and six cases in which the hemoglobin was "saturated" before placing in the chamber.

In sixteen specimens of normal blood which were transferred directly to the microspirometer without being exposed to the air, the average amount of oxygen used was 6.66 cc. per 100 cc. of blood. An example of such an experiment in which the blood was not previously saturated with oxygen is shown in Table I.

In the experiment recorded in Table I the difference between the oxygen content at the beginning of the observations and at the completion of the experiment, as measured by the Van Slyke apparatus, was 5.49 cc. The degree of unsaturation of the blood at the beginning was 6.24 per cent, so that the blood became practically saturated in the microspirometer.

The amount used in 1 hour was 6.72 cc. of oxygen per 100 cc. of

blood. This included the 6.24 cc. to saturate the hemoglobin, so that the actual consumption in this case was 0.48 cc. This is such a small amount that it is hardly significant, as it is but little over twice the possible error which arises in reading the manometer. The results in fifteen other experiments with normal blood under similar conditions were entirely comparable.

In six specimens of normal blood which were "saturated" with oxygen outside of the apparatus the oxygen consumption averaged

TABLE I.  
*Rate of Oxygen Consumption by Blood of Normal Individual.*

Time	Amount of oxygen used by 100 cc. venous blood	Time	Amount of oxygen used by 100 cc. venous blood
min	cc.	min	cc.
0	0	80	8.89
5	0 47	90	10 02
20	2 07	110	11.42
30	3 10	150	11 88
40	5 55	160	14 49
60	6 72	165	15.33
70	8.28	175	15 33

Oxygen content at beginning of observations	17.55 cc. per 100 cc. of blood
" " " end of observations	23.04 " " 100 " " "
Oxygen-combining power	23.79 " " 100 " " "

The microspirometer was in the water bath at 37.5°C. for 1½ hours before the blood was placed in the metabolism chamber. 15 minutes elapsed from the time that the blood was drawn to the first reading on the manometer.

0.055 cc. per 100 cc. of blood. The error in reading the meniscus of the bile tube may represent 0.235 cc. so that the oxygen absorption of normal blood, as determined by this microspirometer, is zero within the limits of experimental error.

#### *Polymorphonuclear Neutrophil Leucocytosis.*

The simplest change in blood that may affect its metabolism is an increase in the number of white blood corpuscles due to leucocytosis. In blood with a high leucocyte count, the absorption of oxygen be-

came appreciable under the conditions of these experiments. Table II shows data concerning the oxygen absorption by this blood from a patient with an infected tumor growth. The white blood cell count was 24,000 per c.mm. and 85.6 per cent of the cells were mature polymorphonuclear leucocytes. The blood was saturated with oxygen for 5 minutes in a large flask. The preliminary increase in volume of the respiration chamber gases, noted in all the experiments and referred to in the preceding paper (3), continued for 60 minutes after the microspirometer was closed. The first decrease in volume was recorded 67 minutes after the blood was drawn.

TABLE II.

*Rate of Oxygen Consumption by Blood with a Polymorphonuclear Neutrophil Leucocytosis.*

(White blood cells 24,000 per c.mm. 20,000 polymorphonuclear neutrophils.)

Time	Amount of oxygen used per 100 cc. oxygenated blood	Time	Amount of oxygen used per 100 cc. oxygenated blood
<i>min.</i>	<i>cc.</i>	<i>min.</i>	<i>cc.</i>
0	0.0	30	0.71
10	0.25	50	1.18
20	0.47	60*	1.41

\* During the next 50 minutes there was no more oxygen absorption as indicated by identical readings of the level of the bile in the manometer.

Two sets of observations on the blood of the same patient on different days gave the following results: When the absolute white blood cell count was 22,250 per c.mm. (19,180 polymorphonuclear neutrophils) the oxygen consumption was 2.75 cc. per hour per 100 cc. of blood. When the count was 18,700 (15,800 polymorphonuclear neutrophils), the oxygen consumption was 1.73 cc. per hour per 100 cc. of blood.

The oxygen content at the end of the experiment was less than that at the beginning, and the hemoglobin was 4.73 per cent unsaturated, even though it had been exposed to oxygen throughout the observations. This was different from the normal bloods and was comparable to the condition in the leucemic bloods described later.

*Chronic Myclogenous Leucemia.*

In blood from patients with chronic myelogenous leucemia, where many of the cells are of an immature and often atypical sort, the rate of oxygen absorption is easily measurable. The observations recorded in Table III illustrate the rate and amount of oxygen absorption in the blood of a woman who had had leucemia for  $2\frac{1}{2}$  years.

TABLE III.

*Oxygen Consumption by Blood of a Patient (No. 5) with Chronic Myclogenous Leucemia.*

Time	Amount of oxygen used per 100 cc. of oxygenated blood	Time	Amount of oxygen used per 100 cc. of oxygenated blood
min.	cc.	min.	cc.
0	0.0	140	18.34
10	0.47	150	19.28
20	1.88	160	20.22
30	3.29	170	21.16
40	4.23	180	23.52
50	5.65	190	25.86
60	6.59	200	27.75
75	7.99	210	30.15
90	10.35	220	32.44
100	12.23	230	34.34
110	13.64	240	34.34
120	15.52	250	36.68
130	17.40	260	37.63

Oxygen content at beginning of observations.....	13.55 cc. per 100 cc. of blood
" " " end of observations.....	2.80 " " 100 " " "
Oxygen-combining power.....	13.55 " " 100 " " "

At the time the tests were made the white blood cell count of the venous blood was 176,000 per c.mm. and only 19.4 per cent of the cells were mature. The rest were grossly immature, chiefly myeloblasts.

The difference between the oxygen content at the beginning of the observations and at the completion of the experiment, as measured by the Van Slyke apparatus, was 10.75 cc. Although the blood was saturated at the beginning, it was very far from saturated at the

end of the observations. The amount of oxygen used by the same sample of blood in the microspirometer during this period was 37.63 cc.

Another portion of the same specimen of blood was kept under oil in an incubator, at 37.5°C. and the oxygen content was deter-

TABLE IV.

*Oxygen Consumption by Blood in Ten Cases of Chronic Myelogenous Leucemia.*

Patient No.	White blood cells per c.mm.	P.M.N.* per c.mm.	Immature cells per c.mm.	Red blood cells per c.mm.	Hemoglobin	Amount of oxygen consumed per hr. per			
						100 cc. whole blood	1000 W.B.C.	1000 mature P.M.N.	1000 immature leucocytes
							$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$
				millions	per cent	cc.	cc.	cc.	cc.
1	37,000	23,700	5,900	2.8	53	4.48	1.210	2.314	7.593
2	52,000	21,600	28,500	3.0	55	3.84	0.730	1.780	1.347
3	122,000	38,300	79,300	3.2	73	9.72	0.796	2.537	1.225
4	123,000	47,900	67,700	3.1	68	12.67	1.036	2.641	1.874
5	176,000	34,100	140,800	3.1	64	7.30	0.414	2.138	0.518
6	454,000	90,800	345,000	3.0	59	18.56	0.408	2.044	0.538
Average.....						9.42	0.765	2.420	2.182
7	71,000	29,100	38,300	2.2	73	6.14	0.864	2.109	1.603
8	80,000	30,200	45,600	4.0		6.46	0.807	2.136	1.416
9	111,000	38,000	54,800	4.1	72	5.89	0.526	1.549	1.076
10	251,000	20,800	230,200	2.9	35	8.77	0.349	4.200	0.381
Average.....						6.815	0.636	2.468	1.119

\* The absolute number of mature polymorphonuclear neutrophils is contrasted with the absolute number of immature cells which include myelocytes of all stages and myeloblasts. The number of eosinophilic and basophilic polymorphonuclear cells is not included. The number of lymphocytes, which is comparatively small in most leucemic bloods with high white counts, is disregarded in this comparative study.

mined at intervals by Dr. Arlie V. Bock. Practically all the oxyhemoglobin was reduced in 3 hours. The oxygen-combining power at the start was 13.55 cc. per 100 cc. of blood, and at the end was 0.04 cc. per 100 cc. of blood.

The data of the first six cases of chronic myelogenous leucemia

given in Table IV are better for comparative purposes than the other four cases because the red blood counts (2.8 to 3.2 millions per c.mm.) and the hemoglobin percentages (53 to 68, Sahli) were similar enough to be practically the same. The color indexes of these six patients were: 0.94; 0.91; 1.1; 1.09; 1.03; and 0.98 (average 1.008) while those of the others were 1.6; 0.87; and 0.61. The influence of the number of red blood corpuscles and the per cent of hemoglobin thus did not enter significantly into variations in oxygen absorption by the first six cases enumerated so that it is proper to attribute variations in metabolic activity especially to the leucocytes.

#### DISCUSSION.

When blood from a normal individual is drawn from a vein and transferred directly to the microspirometer, part of the oxygen absorbed is used to convert the reduced hemoglobin to oxyhemoglobin. The amount used in excess of this represents, to a great extent, the oxygen absorbed by the leucocytes. The reticulated red blood cells (young cells) apparently have the power to absorb oxygen (Morawitz (5), Morawitz and Itami (6), Harrop (7), and Denecke (8)). In the cases of leucemia with a high white blood cell count they formed but a minor fraction of the total number of metabolizing cells, so that the amount of oxygen that they used was but a small part of the total. The part played by the blood platelets in the consumption of oxygen is not known.

In normal blood, when the hemoglobin is saturated, the oxygen consumption is less than in blood in which the number of leucocytes is high. Furthermore, the process of utilization of oxygen lasted longer in the leucemic blood and that from the patient with leucocytosis than in a normal blood. The readings on the manometer tube usually became constant after  $1\frac{1}{2}$  hours when normal blood was being studied, while a constant progressive change was noted in the bloods with high white counts from  $1\frac{1}{2}$  to over 10 hours, and possibly longer. Leucemic blood used from 3.7 cc. to 70.5 cc. of oxygen per 100 cc. of blood during the course of the period of observation (*i.e.* until absorption stopped or the experiment was discontinued) while there was practically no absorption by normal blood and 1.41 cc. to 1.76 cc. by the blood of the patient with leucocytosis, when absorption was complete.



When the different leucemic bloods and the one with simple leucocytosis are compared, and an effort is made to correlate the amount of oxygen used per hour with the total white blood count, the total number of immature white blood cells, the total red blood cell count, the hemoglobin percentage, and the total number of mature polymorphonuclear neutrophils, the highest degree of correlation is found with the last named factor. This is shown in Chart 1. In

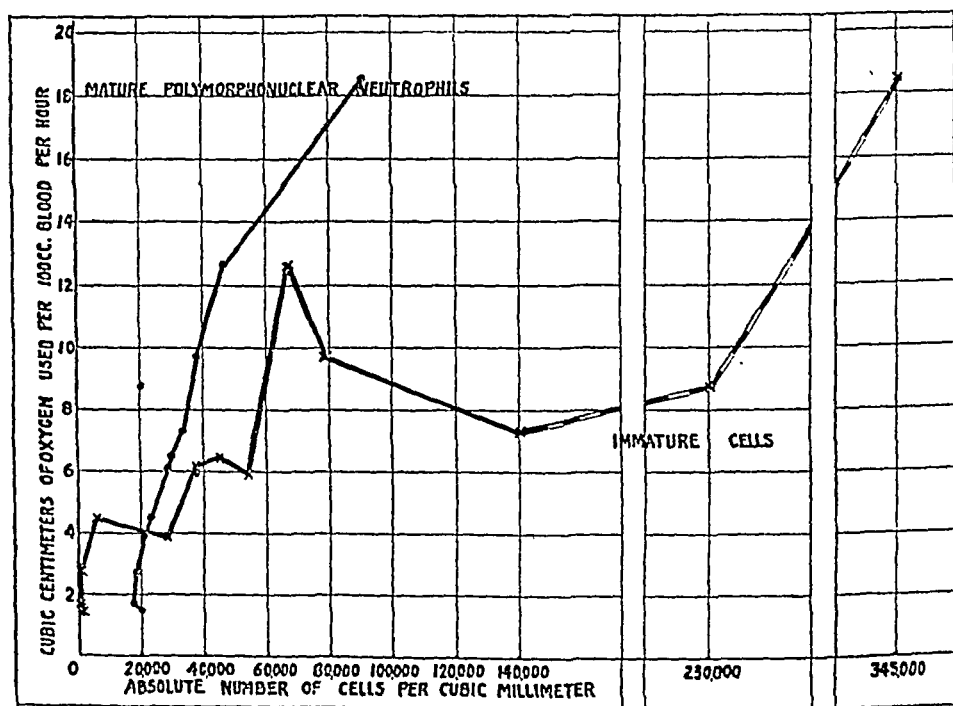


CHART 1. Rate of oxygen consumption by 100 cc. of blood compared with the absolute number of mature polymorphonuclear neutrophils and with the number of immature cells in sepsis and in chronic myelogenous leucemia.

Table IV it will be noted that the individual observations vary from the average the least when it is assumed that the bulk of the oxygen absorbed is taken up by the mature polymorphonuclear neutrophils, and the variation from the average is much greater when the total white blood cell count or the number of immature cells is used for comparison. The adult cells appear to use more oxygen than the immature ones under the conditions of these experiments as shown

in Table IV. In Patient 5 the number of immature cells was 176,000 per c.mm., whereas in Patient 4 the number of immature cells was 67,650 per c.mm. In the former the rate of oxygen absorption, under the conditions of this microspirometer, was only 7.30 cc. per hour per 100 cc. of blood whereas in the latter the consumption was 12.67 cc. per hour. Evidently the immature cells used less oxygen than a corresponding number of mature cells. Whether this is true of the cells in the body or not is a matter for additional study. The immature cells may be more susceptible to changes in their environment than the adult cells, and therefore show a lower rate of metabolism *in vitro*.

If the rate of metabolism of the immature white blood cells is low, as compared with that for the adult cells, the former resemble tumor cells in this respect more than embryonic or young tissue cells. The immature leucocytes behaved as did Warburg's tumor tissue (1) when exposed to the conditions of a microspirometer. These observations also make it seem improbable that there is a direct relation between the oxygen metabolism of the immature blood cells in the peripheral circulation and the increased basal metabolic rate of the leucemic patients. The hemoglobin of normal blood, as noted after it has been for a long period of time in the microspirometer, remains saturated with oxygen. This is sharply in contrast to what happened under similar circumstances to the leucemic blood, for it was found that its hemoglobin was no longer saturated with oxygen, although it was exposed to the air of the respiration chamber and kept in motion. When such blood was taken from the apparatus and exposed to the air of the room it was found that it did not take up oxygen to the full capacity of the hemoglobin present, so that in some cases it was as much as 4.78 volumes per cent desaturated. As this was a fairly constant observation for leucemic blood when the white blood cell count was high, and not characteristic of normal blood as noted by us and by others (Harrop (7)), it is thought that there may have been some factor which changed part of the hemoglobin. However, there was no definite evidence of this and spectroscopic examination failed to show the presence of methemoglobin.

The carbon dioxide was absorbed in all these experiments as fast as it was produced. Evidently some "fixed" acid, probably lactic,

was formed as in those experiments on leucemic blood in which the carbon dioxide-combining power of the plasma was measured before and after the observations. It was found that the carbon dioxide-combining power was reduced in the leucemic bloods to practically nothing as the figure obtained was so small that it was within the limits of possible error of the method of determination.

There were no gross changes noted in general morphologic characters of the cells after they had been in the microspirometer for the period of the experiments. They appeared normal in all respects when studied with supravital stains or examined in films colored with Wright's stain. The oxidase reaction was the same or possibly more intensely positive at the end than at the beginning of the experiment.

#### SUMMARY AND CONCLUSIONS.

1. The oxygen consumption of blood of normal individuals, when the hemoglobin is saturated with oxygen, is practically zero within the limits of experimental error of the microspirometer used.

2. The oxygen consumed in a microspirometer by the blood of patients with chronic myelogenous leukemia with a high white blood cell count, and of one with leucocytosis from sepsis, was proportional to the number of adult polymorphonuclear neutrophils in the blood.

3. No correlation could be made between the rate of oxygen absorption and the total number of white blood cells in the blood, or the total number of immature cells, or the number of red blood cells, or the amount of oxyhemoglobin.

4. The blood of patients with chronic myelogenous leukemia continued to use oxygen in the microspirometer longer than that of normal individuals, and the hemoglobin, in the leucemic bloods, became desaturated even though exposed to air.

5. In blood in which the bulk of the cells were immature and the mature cells few, the oxygen consumption was lower than in blood in which the mature cells predominated. The rate of oxygen consumption of the immature cells was relatively low as compared to the mature.

6. The slower rate of oxygen absorption by the immature leucocytes in chronic myelogenous leukemia as compared to the mature cells, places them, in accord with Warburg's reports, in the class of

the malignant tissues in this respect rather than in the group of young or embryonic cells.

Throughout the course of this work we have received advice and criticism from various associates. We wish to express our appreciation for their assistance, especially to the following: Dr. George R. Minot, Dr. Lawrence J. Henderson, Dr. Thorne M. Carpenter, Dr. James H. Means, and Dr. Blake C. Wilbur.

#### BIBLIOGRAPHY.

1. Warburg, O., The metabolism of the cancer cell, *Cancer Rev.*, 1926, i, 298; Über den Stoffwechsel der Carcinomzelle, *Klin. Woch.*, 1925, iv, 534; Das Carcinomproblem, *Z. angew. Chem.*, 1926, xxxix, 949.
2. Riddle, M. C., and Sturgis, C. C., Basal metabolism in chronic myelogenous leukemia, *Arch. Int. Med.*, 1927, xxxix, 255.
3. Wilbur, B. C., Daland, G. A., and Cohen, J., Cell respiration studies. I. A microspirometer for the continuous study of the oxygen absorption by living cells, *J. Exp. Med.*, 1927, xlv, 43.
4. Lundsgaard, C., and Möller, E., On the determination of the total oxygen-combining power of the blood in the Van Slyke apparatus, *J. Biol. Chem.*, 1922, lli, 377.
5. Morawitz, P., Über Oxydationsprozesse im Blut, *Arch. exp. Path. u. Pharmacol.*, 1909, lx, 298.
6. Morawitz, P., and Itami, S., Klinische Untersuchungen über Blutregeneration, *Deutsch. Arch. klin. Med.*, 1910, c, 191.
7. Harrop, G. A., The oxygen consumption of human erythrocytes, *Arch. Int. Med.*, 1919, xxiii, 745.
8. Denecke, G., Ueber den Stoffwechsel der Erythrozyten im anämischen Blute. *Deutsch. med. Woch.*, 1926, lii, 280.



# EFFECT OF HOST IMMUNITY TO A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE RABBIT NEOPLASM.

By LOUISE PEARCE, M.D., AND THOMAS M. RIVERS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 22, 1927.)

In a previous paper (1) experiments were reported in which it was shown that 2 filterable viruses, Virus III and vaccine virus, grow and survive for unusually long periods in a transplantable malignant neoplasm of the rabbit. Early in the course of this work, it was found that the tumor (2) was infected with Virus III (3) and was being transferred with it, and also that the rabbits so inoculated became immune to Virus III. Since Virus III is indigenous to rabbits and its incidence fairly high judging from the number of immune animals in the normal stocks of these laboratories (15 to 20 per cent on an average), it is impossible to say when the tumor became infected. In any event, the question arose as to whether growth and malignancy of the tumor were affected first, by the presence of the virus, and second, by host immunity to the virus. An opportunity to investigate these problems was afforded by the fact that we were able to obtain the tumor without the virus and to transplant it in the same manner as the stock tumor bearing the virus.

Experiments are reported in the present paper dealing with the effect of the immunity of the host to Virus III on the pathological processes induced by both the virus-bearing and the virus-free tumor strains. A subsequent paper deals with the question of the effect of the virus on the growth and malignancy of the tumor (4).

## EXPERIMENTAL.

### *Materials and Method.*

*Neoplasm.*—The tumor employed in these experiments has been studied extensively ever since its recognition in October, 1920, and its subsequent trans-

plantation, and a number of papers dealing with various aspects of the subject have been published (2). It suffices to state here that the tumor is considered to be of epithelial origin composed of cells allied to those found in the bulb and root sheath of the hair, and that it has been transplanted through successive generations by intratesticular inoculations. This method of inoculation has proven effective, not only from the standpoint of obtaining actively progressing primary tumors, but also for the study of the pathological process as a whole. The manifestations of the disease vary markedly both in individual rabbits of a series and between groups of animals inoculated at one time as compared with those inoculated at another. The growth and fate of the primary tumor, the incidence of metastases, the distribution, number, extent and state of the growths and the mortality rates, both actual and estimated, are among the variable features of the disease which must be taken into account in evaluating the character of the process at any time.

It was found in October, 1924, that all rabbits inoculated with the tumor became immune to Virus III, and by means of rapid passage of emulsions of primary tumors or metastases, the presence of the virus was regularly demonstrated.

*Virus III.*—While attempting to produce chicken-pox in rabbits, a filterable transmissible agent was discovered (3). This agent produces gross as well as microscopic lesions in the cornea, skin and testicles of rabbits, and an infection with it leads to an immunity against subsequent infections with the same material. For convenience, this agent has been spoken of as Virus III. At first it was considered not unlikely that the virus is the etiological agent of varicella. Further work, however, disclosed the fact that Virus III is indigenous to rabbits and that it is as typical a virus as vaccine virus or the virus of herpes simplex from both of which it can easily be differentiated.

*Virus-Free Strain of Tumor.*—This strain was obtained from a rabbit inoculated with the stock tumor bearing the virus. The animal died several weeks after inoculation and accidentally lay in a warm room 12 to 18 hours. The primary tumor was removed and inoculated into the testicles of 3 rabbits. Although the development of these transplants was much delayed, growth later occurred and transfers were successfully made. Many generations of this strain of the tumor have been studied, and it has been impossible to demonstrate the presence of Virus III by methods which suffice with the stock tumor. Rabbits inoculated with this strain, moreover, do not become refractory to skin infection with Virus III, their sera do not become virucidal and no nuclear inclusions, typical of Virus III reactions, have been found in young tumors. The virus-free state of this strain was controlled from time to time by appropriate tests.

*Conduct of Experiments.*—The experiments were carried out from February, 1925, to January, 1926. Groups of 10 male rabbits from selected stocks were immunized to Virus III by a single intracutaneous or subcutaneous injection of testicular emulsions containing the virus. At intervals of 20 to 39 days after the injection of Virus III, the rabbits were inoculated in one testicle with 0.3 cc. of a

salt solution emulsion of an actively growing primary tumor. Comparable groups of non-immunized rabbits were inoculated at the same time. Both the stock tumor strain bearing the virus and the virus-free strain of tumor were used. The total number of rabbits employed in the experiments reported in this paper was 129, 49 of which were immunized.

The rabbits were separately caged and fed the same diet of hay, oats and cabbage. Frequent examinations were made, special attention being paid to the general physical condition of the animals, the character and course of the primary tumor and the development of secondary growths in superficial parts of the body.

The experiments were terminated 2 months after inoculation at which time all surviving animals were killed by an injection of air in the marginal ear vein. This period was selected upon the basis of previous experience as being sufficient to include a large proportion of the deaths due to tumor growth, and at the same time, sufficient to allow for the recovery of many rabbits. Rabbits which developed a pronounced cachexia or paralyzes during the observation period were killed at that time. Each animal was subjected to postmortem examination, particular attention being given to the state of the primary tumor and to the distribution, number and condition of secondary growths together with an estimation of the degree of organ involvement.

*Method of Analysis of Results.*—The data obtained from clinical observations and postmortem examinations have been analyzed upon a group basis. The actual deaths from the tumor process have been classified in 2 groups upon the basis of postmortem findings. In one, designated as "malignant," the widespread or significant distribution of tumor was such that there could be no question that the malignant process was responsible for the death of the animal. In the other, designated as "accidental," the distribution of tumor was usually more limited, and except that a site such as the spine or jaws was involved, it has been assumed that death would not have occurred at this time. It is obvious that in an estimation of degrees of malignancy based upon comparative mortality rates, the numbers of accidental fatalities possess far less significance than those in the category of malignant deaths.

A considerable number of rabbits survived the observation period of 2 months. In some of them, however, the distribution of metastases was such that it is probable that death would eventually have occurred as a result of the tumor process. Growths in both suprarenal glands or in the facial and jaw bones are instances of this type of disease. These cases have been classified as "probable deaths." On the other hand, there were instances of surviving animals in which a few foci of tumor were found, but upon the basis of the distribution and state of these growths, they have been classified as "probable recoveries."

The number of foci of metastases refers to the number of organs or tissues involved, not to the actual numbers of secondary growths, and consequently, the expressions "foci of metastases," "distribution of metastases" or "metastatic rate" are used rather than "number of metastases."



TABLE I.  
*Virus-Free Tumor in Normal and Immune Rabbits.*

Experiment	Group	Time after Virus III infection	No. of rabbits	Primary tumors	Mortality						Metastases				No. of metastatic foci		Probable deaths	Actual and probable recoveries			
					Total		Malignant cases		Accidental deaths		Incidence		Total foci							Relative rate	
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent				No.	Per cent
I	N		9	All +	3	33.3	2	22.2	1	11.1	6	66.7	62	6.9	10.3	14, 20, 22	3, 2,	0 in 5	1	5	55.5
	I	39	9	All +	1	11.1	1	11.1	0	—	3	33.3	23	2.6	7.7	19	2, 2	0 in 6	1	7	77.7
II	N		10	All +	1	10.0	1	10.0	0	—	6	60.0	41	4.1	6.8	25	4, 4, 3, 3, 2	0 in 4	1	8	80.0
	I	25	9	8 +	1	11.1	0	—	1	11.1	5	55.5	21	2.3	4.2	11	7, 1, 1, 1	0 in 4	1	7	77.7
III	N		10	All +	1	10.0	1	10.0	0	—	4	40.0	32	3.2	8.0	21	8, 2, 1	0 in 6	1	8	80.0
	I	28	11	All +	0	—	0	—	0	—	6	55.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	10	100.0
Total	N		29		5	17.2	4	13.8	1	3.4	16	55.2	135	4.7	8.4	102	32	0 in 15	4	20	68.9
	I		29		2	6.9	1	3.4	1	3.4	14	48.3	64	2.2	4.6	30	34	0 in 15	3	24	82.7

N = normal rabbits; I = rabbits immune to Virus III.

TABLE II.  
*Virus-Bearing Tumor in Normal and Immune Rabbits.*

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumors	Mortality						Metastases				No. of metastatic foci		Probable deaths	Actual and probable recoveries				
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths		Survivors		No.	Per cent	
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent										
IV	N	10	10	+	+	4	40.0	4	40.0	0	—	6	60.0	101	10.1	16.8	30, 32, 16, 12	9, 2	0 in 4	1	5	50.0
	I	28	10	+	+	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	6.7	25, 6	4, 2, 2, 1	0 in 4	0	8	80.0

N = normal rabbits; I = rabbits immune to Virus III.

TABLE III.  
*Virus-Bearing Tumor in Normal Rabbits. Virus-Free Tumor in Immune Rabbits.*

Experiment	Group	Time after Virus III infection	No. of rabbits	Primary tumors	Mortality				Metastases				No. of metastatic foci		Probable deaths	Actual and probable recoveries			
					Total		Malignant cases		Accidental deaths	Incidence		Total foci	Relative rate	Actual rate		Deaths	Survivors	No.	Per cent
					No.	Per cent	No.	Per cent											
V	N	days	10	All +	550.0	440.0	1	10.0	770.0	74	7.4	10.6	21, 17, 16, 9, 7	3, 1	0 in 3	0	5	50.0	
	I	39	9	All +	111.1	111.1	0	—	333.3	23	2.6	2.6	19	2, 2	0 in 6	1	7	77.7	
VI	N	10	10	All +	550.0	330.0	2	20.0	770.0	75	7.5	10.7	21, 15, 14, 14, 6	3, 2	0 in 3	0	5	50.0	
	I	20	10	9 +	110.0	0	1	10.0	110.0	10*	1.0	10.0	10		0 in 9	0	9	90.0	
VII	N	11	11	All +	436.4	327.3	1	9.1	981.9	74	6.7	8.2	26, 19, 14, 6†	4, 2, 1, 1, 1	0 in 2	0	7	63.6	
	I	25	9	8 +	111.1	0	1	11.1	555.5	21	2.3	4.2	7	11, 1, 1, 1	0 in 4	1	7	77.7	
VIII	N	10	10	All +	440.0	440.0	0	—	660.0	101	10.1	16.8	32, 30, 16, 12	9, 2	0 in 4	1	5	50.0	
	I	28	11	All +	0	0	0	—	655.5	20	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	11	100.0	
Total	N		41		1843.9	1434.1	4	9.8	2970.4	324	7.9	11.0	70	29	0 in 12	1	22	53.7	
	I		39		37.7	12.6	2	5.1	1538.5	74	1.9	4.9	36	37	0 in 24	3	33	84.6	

N = normal rabbits; I = rabbits immune to Virus III.

\* Number of metastatic foci estimated. † Complicating empyema.

TABLE IV.  
*Virus-Free Tumor in Normal Rabbits. Virus-Bearing Tumor in Immune Rabbits.*

Experiment	Group	Time after Virus III injection	No. of rabbits	Primary tumor	Mortality						Metastases				No. of metastatic foci		Probable deaths	Actual and probable recoveries		
					Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate	Actual rate	Deaths		Survivors	No.	Per cent
					No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent								
IX	N	28	10	All +	1	10.0	1	10.0	0	—	4	40.0	32	3.2	6.7	21	8, 2, 1	0 in 6	9	90.0
	I	28	10	All +	2	20.0	1	10.0	1	10.0	6	60.0	40	4.0	8.0	25, 6	4, 2, 2, 1	0 in 4	8	80.0

N = normal rabbits; I = rabbits immune to Virus III.

The distribution of secondary growths has been considered upon both a relative and an actual basis, the former including all animals of a group, while the latter takes into account only those rabbits in which metastases were found. The actual rate is obviously accentuated by individual rabbits with large numbers of foci, so that from a group standpoint the relative rate is a fairer index of comparative metastatic involvement. For other purposes, such as a numerical comparison of the uniformity of tumor distribution, both rates are of value.

### *Results.*

The results of 9 experiments consisting of both clinical and post-mortem observations are summarized in Tables I, II, III and IV.

Table I contains data obtained in 3 experiments in which the virus-free tumor was inoculated in normal rabbits and in rabbits injected 25, 28 or 39 days previously with Virus III. Table II consists of the data of one experiment in which the virus-bearing tumor was used in an immunized group of rabbits; Virus III had been injected 28 days before the tumor. In the next 4 experiments, Table III, the pathological picture in normal rabbits induced by the virus-bearing tumor is contrasted with that of the virus-free tumor in rabbits injected 20, 25, 28 or 39 days previously with Virus III. Finally, Table IV summarizes the results of a single experiment in which the behavior of the virus-free tumor in normal rabbits was compared with that of the virus-bearing tumor in rabbits injected 28 days previously with Virus III.

### DISCUSSION.

Before discussing the results of the experiments in which the course and character of the malignant disease in rabbits immune to Virus III was investigated, certain features of this study which must be considered in interpreting and evaluating the results should be briefly mentioned.

Because of the variability in the manifestation of the disease induced by this tumor, and in particular, the seasonal character of these variations (5), it is important in an investigation of comparisons of the disease under diverse conditions to carry out experiments at different seasons of the year. This has been done in the present instance as shown by the following dates of tumor inoculation:

Experiments I and V.....	March 13 and 18, 1925.
Experiment VI.....	May 12 and 15, 1925.
Experiments II and VII.....	October 27 and 28, 1925.
Experiments III, IV, VIII and IX.....	November 24 and 25, 1925.

The state of different materials used for inoculation must be considered in comparing results of 2 series, one of which was inoculated with the tumor bearing the virus and the other with the virus-free tumor (Tables III and IV). Both strains were transferred at monthly intervals to groups of not less than 10 rabbits and although actively growing primary tumors were used for this purpose, there was no criterion which would enable one to say that the 2 tumors were alike in actual or potential qualities of growth. But the chance of using less favorable material was the same in one case as in the other, except for the fact, which will be discussed later, that the disease in rabbits immune to Virus III was less malignant than in normal animals, and in certain experiments the virus-free tumor used for inoculation was derived from the primary growth of this strain in an immune rabbit. The possibility that this condition of host immunity modified the growth capacity and malignant potentialities of the tumor cannot be disregarded, but on the other hand, it should be pointed out that there is no reason for assuming that such modifications were of the nature of fixed characteristics.

One must also consider the interval between the inoculation of the normal and of the immune groups of an experiment. This factor does not enter into the first 4 experiments in which both groups were inoculated on the same day or in the last 3 in which inoculations were carried out on succeeding days. But in the 5th experiment there were 5 days, and in the 6th experiment, there were 3 days intervening between the inoculation of the 2 groups. What effect, if any, such a time difference might have upon the course and character of the malignant disease cannot be predicted, and one might be disposed to ignore it because of its shortness as compared with the 2 months' duration of the experiments and attribute any marked divergence of the pathological picture in the 2 groups to other factors. We have repeatedly observed, however, definite variations in the plane or level of malignancy in series of rabbits inoculated at intervals of 2 weeks, and in some instances of 1 week, when the material used for inoculation was

apparently as favorable in one case as in the other and other conditions under experimental control were common to both sets of animals. On this account, a number of experiments in which the interval between the inoculation of the 2 groups was 1 week or longer have not been included in the present paper. It may be said, however, that the observations derived from these additional experiments are in general agreement with those reported.

The analysis of experimental data may conveniently begin with the first result of inoculation, namely, the primary tumor. It has been our experience with the intratesticular route of injection that a primary tumor rarely fails to develop. In the present series of normal rabbits no failures were observed, but in 2 of the immune animals no tumor appeared. Although this proportion of failures is small, and may be entirely due to an error in the technic of inoculation, it is of interest that they occurred in the immune and not in the normal series. No attempt was made to measure the rate of growth and ultimate size of the primary tumors nor the speed and extent of regression in the instances in which this change occurred, but the general impression obtained of the initial reaction was that the tumors tended to develop more slowly and more irregularly in immune than in normal rabbits.

It will be seen by reference to Table I that in 3 experiments the disease was considerably less severe in rabbits immunized to Virus III than in normal animals; the virus-free strain of the tumor was used in these experiments. The lower level of malignancy in the immune group of each experiment is clearly brought out by the lower mortality rate, the fewer instances of pronounced malignancy, the smaller number of metastatic foci, the lower relative and actual rates of these growths and the higher incidence of actual and probable recoveries. As far as incidence of metastases is concerned, there is no consistent difference in the 3 experiments, but if the data are combined, the incidence is slightly lower among the immune than among the normal rabbits, that is, 48.3 per cent as contrasted with 55.2 per cent.

One experiment is available in which the behavior of the virus-bearing tumor was studied in 10 normal rabbits and in 10 rabbits immunized to Virus III (Table II). Again, the disease was much milder in the immunized group. The mortality rate was only half as

great, and the incidence of well marked cases of malignancy was one-fourth as high as in the group composed of normal animals. There was a much lower number of metastatic foci with a consequent reduction in the relative rate of these growths and although there was no difference in the incidence of metastases, the actual rate, in which only the animals with metastases are considered, was much smaller in the immune group, that is, 6.7 as compared with 16.8 in the normals.

The next comparison has been made with the virus-bearing tumor in normal and the virus-free tumor in immunized rabbits (Table III). The results of the 4 experiments are in general conformity with the others, but the contrast between the level of malignancy displayed by the normal groups and the very mild character of the disease of the immune series is even more pronounced. In each experiment, for instance, there were 3 or 4 cases of outspoken malignancy among the normal groups, but there was only 1 such case among all the immunes of the 4 experiments. There was also a much lower incidence of metastases in 3 immune groups, while in the 4th (Experiment VIII) it was slightly lower. As far as the numbers of metastatic foci and the relative and actual rates of these growths are concerned, the values for the immune groups are uniformly smaller than those for the normals.

In the last experiment, observations of immune rabbits inoculated with virus-bearing tumor are compared with those of normal rabbits inoculated with virus-free tumor (Table IV). The results of this experiment are not in accord with the others. Thus, there was the same incidence of pronounced cases of malignancy and practically the same number of metastatic foci with comparable relative rates of secondary growths in both immune and normal groups. In 2 particulars, however, the disease of the immune group was more severe than that of the normals, namely, in the higher incidence of metastases and in the slightly smaller number of actual and probable recoveries. But the disease of the immune group was considerably less severe than that of a group of normal rabbits inoculated with the same material (Experiment IV, Table II), so that as far as the reaction of the host to this particular inoculum was concerned, the resistance of rabbits immune to Virus III was more effective than that of normal



animals. It is probable, therefore, that the discordant results of the experiment were associated with the other group of animals, namely, the normal rabbits inoculated with virus-free tumor. The disease which developed in these animals was very mild, but not as mild as in a group of immune rabbits inoculated with the same material (Experiment III, Table I). It is likely, therefore, that the particular results obtained in Experiment IX were largely influenced by the character of the virus-free material used for inoculation. In the earlier work with the virus-free tumor the strain was carried in normal rabbits, but later, Virus III immune animals were used because it was feared that a reinfection of the tumor might occur. During the period of these experiments, the same method of monthly transfer of the virus-free strain in immune rabbits and of the virus-bearing stock tumor in normal rabbits was employed, but the fact that the disease in immune animals was comparatively mild suggests that material from such sources might not be as favorable as transplants taken from normal rabbits. That is to say, from the standpoint of the animal factor, conditions tended to favor growth and development of the tumor in the case of the virus-bearing strain, while the reverse obtained with the virus-free strain. On the other hand, it is important to note that the virus-free strain after a sojourn in immunized animals was still capable of inducing a process of well marked malignancy, for there were 2 such occurrences in the normal rabbits of Experiments II and III (Table I). It would appear, therefore, that the comparable results obtained with the immune and normal groups of Experiment IX were due, not to the failure of the immune state as such to be associated with a comparatively mild disease, but to the low level of the malignant process which developed in the normal rabbits.

Finally, as a conclusion to the comparison of individual experiments, the data of 7 experiments (I, II, III, IV, VI, VIII and IX) have been combined. The other 2 experiments cannot be used for this purpose because the data of their immune, although not of their normal groups, appear in other experiments. The observations are derived from 69 normal and 70 immune rabbits and have been analyzed in the same manner as in individual experiments with the following results:

	Total deaths	Malignant cases	Metastases			
			Incidence	No. of foci	Relative rate	Actual rate
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
Normals.....	27.53	23.19	56.50	444	6.43	11.37
Immunes.....	10.00	4.29	47.15	174	2.48	5.27

The result of this analysis brings out in a striking manner the fact that the character of the malignant process in rabbits immunized to Virus III was much less severe than in normal rabbits, and further, that this lowered plane of malignancy occurred despite relatively little difference in the incidence of metastases. Virus III immunity did not diminish the number of rabbits in which metastases were found, but the unfavorable effect of this state upon the disease was evidently exerted upon the development of certain of these secondary growths as shown by the total number of foci, together with their relative and actual rates. But this unfavorable effect did not invariably occur, for there were 2 instances of pronounced malignancy in 2 immune groups (Experiments I and IV), a ratio of, roughly, 1 in 10. It is evident, therefore, that the reaction of the exceptional animal is little, if at all, influenced by the presence of an immunity to Virus III, and the inclusion of these rabbits disturbs the tendency toward numerical uniformity of pathological manifestations otherwise obtaining in animal groups in which the tumor process is of low malignancy.

The question of the comparative effectiveness of the immune state of rabbits to Virus III with respect to its duration cannot be properly discussed at this time because of insufficient material. Rabbits injected with Virus III become refractory to subsequent injections of the virus (intradermal) within 6 to 8 days, and the sera of such animals show well marked virucidal properties within a fortnight. As far as is known, these conditions continue for at least 6 months. The present experiments were performed 20 to 39 days after the injection of Virus III when a high state of immunity to the virus was present, but it is impossible to say whether variations in the degree of immunity associated with different periods of duration, if such variations exist, could be satisfactorily demonstrated by means of the malignant disease.

Finally, attention should be drawn to the fact that the immunity to Virus III which follows the injection of the virus-bearing tumor does not appear to be associated with an unfavorable influence upon the malignant process. The time necessary for the development of an immunity under these circumstances may account for this result. In the case of rabbits inoculated with testicular tissue emulsions rich in virus content, the immune state is fully developed within 6 to 8 days, but this period is lengthened to 2 to 3 weeks after the injection of the virus-bearing tumor. This difference in time is presumably due to differences in the amount or state of the virus. Under circumstances of rapid testicular passage at 4 or 5 day intervals, the amount of active virus must be very large, while it is undoubtedly smaller in the case of the tumor transferred at monthly intervals as shown by the fact that although testicular inoculations of stock tumors 4 to 8 weeks old lead to an immunity to Virus III, no visible virus reaction is obtained by means of intracutaneous inoculation of the same material.

Since the findings of the experiments reported in this paper show that the tumor process was not as mild in normal rabbits inoculated with virus-bearing tumor as in comparable groups of rabbits in which a Virus III immune state was present at the time of inoculation, it would appear that the character of the tumor process as a whole was largely determined by conditions or factors obtaining during the first 2 weeks after inoculation. As far as this particular tumor is concerned, however, such an assumption is not entirely warranted. For instance, young primary tumors which have grown slowly and to a limited extent for the first 2 or 3 weeks may suddenly assume an active and rapid growth. It is reasonable to presume that a similar change takes place in any metastatic growths with the result that what was apparently a more or less controlled tumor process became an uncontrolled one. The balance which exists between the ability of the host to control the malignant disease on the one hand, and the capacity of the tumor process toward the continued growth of primary and metastatic tumors on the other, is obviously influenced by a variety of factors. Nevertheless, it would appear from the present experiments that the growth capacities of the virus-bearing transplant were not affected by a slowly developing immunity to Virus III in the same manner as in

the case of an immunity present at the time of inoculation. On the other hand, the failure of the tumor process to be influenced by a delayed virus immunity may be explained upon the basis of the effect which the virus exerts on the animal host. This aspect of the question is discussed in the accompanying paper dealing with the effect of Virus III on the malignant disease (4).

The effect of host immunity to Virus III upon the manifestations of this malignant tumor must be of an entirely non-specific nature. Virus III has been extensively studied in a large number of rabbits for 4 years and there is no indication that it produces tumors of any type. Although the stock tumor with which we have worked was found to be infected with Virus III, there is no reason for assuming that its presence was anything but a fortuitous occurrence and due to 2 factors, first, that this virus is indigenous to rabbits and second, that the tumor presents unusually favorable conditions for the growth and survival of certain viruses. Furthermore, a virus-free strain of the tumor has been found to possess the essential characteristics of malignancy exhibited by the virus-bearing strain, for it can be transplanted from rabbit to rabbit, it gives rise to metastatic growths and it has caused death. The effect of Virus III immunity upon the course and character of the neoplasm has, moreover, been observed in the disease induced by both virus-free and virus-bearing tumors. And it may be mentioned in this connection that similar effects have been observed in connection with concomitant infections which have affected the course of the tumor and also experimental infection with *Treponema pallidum*.\*

The mechanism by which this effect is produced is not known. If one considers resistance or susceptibility to disease as a functional activity of the animal organism, then it is evident that the low plane of malignancy displayed by rabbits immunized to Virus III was brought about by factors which affected animal economy resulting in an increased or a more effective resistance to the tumor process.

#### SUMMARY AND CONCLUSIONS.

Experiments are reported in which were studied the course and character of a transplantable malignant neoplasm in normal rabbits and in rabbits immunized with a filterable virus, Virus III.

\* Unpublished experiments of L. Pearce.

The disease which developed in immunized rabbits was extremely mild and much less severe than in normal animals.

The effect upon the tumor process displayed by Virus III immune rabbits in the direction of diminished malignancy is considered to be entirely non-specific in character, and the suggestion is made that it is accomplished through a more effective resistance of the host.

#### BIBLIOGRAPHY.

1. Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, xlii, 523.
2. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 601.  
Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxvii, 631.  
Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 799.  
Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxvii, 811; xxxviii, 347, 367.  
Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxviii, 385.  
Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 583.  
Pearce, L., Brown, W. H., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 603.
3. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673; 1924, xxxix, 777; xl, 281.
4. Pearce, L., and Rivers, T. M., *J. Exp. Med.*, 1927, xlvi, 81.
5. Pearce, L., Brown, W. H., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 603.  
Brown, W. H., Pearce, L., and Van Allen, C. M., *Tr. Assn. Am. Phys.*, 1924, xxxix, 351.

# EFFECT OF A FILTERABLE VIRUS (VIRUS III) ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

BY LOUISE PEARCE, M.D., AND THOMAS M. RIVERS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 22, 1927.)

In connection with the study of the effect of an immunity to Virus III on the course of a transplantable neoplasm of the rabbit (1), other problems relating to the tumor were investigated. The finding that the stock tumor (2) was infected with Virus III (3) which was being regularly transferred with it, suggested the question whether the presence of Virus III affected the growth and malignancy of the tumor. The fact that we were able to obtain the tumor without the virus and to transfer it from rabbit to rabbit furnished the means for an experimental study of this problem, the results of which are reported in the present paper.

## EXPERIMENTAL.

### *Materials and Method.*

The general plan of the experiments consisted of the study of parallel series of rabbits inoculated with the virus-bearing and the virus-free strains of the tumor. The dates of inoculation were as follows:

Experiment I.....	January 14 and 17, 1925.
Experiment II.....	February 16, 1925.
Experiment III.....	March 13 and 18, 1925.
Experiment IV.....	April 15 and 17, 1925.
Experiment V.....	October 27 and 28, 1925.
Experiment VI.....	November 24 and 25, 1925.
Experiment VII.....	November 24 and 25, 1925.

*Neoplasm.*—The tumor which is described fully in earlier papers (2) is considered to be of epithelial origin.

*Virus III.*—This has been described in the preceding paper (1) and more detailed accounts have previously been published (3).

TABLE I.  
*Comparison of Virus-Bearing and Virus-Free Tumor in Normal Rabbits.*

Experiment	Group	No. of rabbits	Primary tumors	Mortality						Metastases				No. of metastatic foci		Probable deaths	Actual and probable recoveries			
				Total		Malignant cases		Accidental deaths		Incidence		Total foci	Relative rate		Actual rate		Deaths	Survivors	No.	Per cent
				No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent									
I	V +	10	All +	220.0	0	—	—	220.0	4	40.0	26	2.6	6.5	10, 7	5, 4	0 in 6	1	7	70.0	
	V -	9	All +	666.6	222.2	444.4	—	444.4	9	100.0	61	6.8	6.8	15, * 11, 9, 7, 5, 3	8, 2, 1	0 in 0	1	2	22.2	
II	V +	9	All +	444.4	444.4	0	—	—	5	55.5	81	9.0	16.2	30, 17, 15, 15	4	0 in 4	0	5	55.5	
	V -	9	All +	666.6	222.2	444.4	—	444.4	6	66.6	70	7.7	11.7	22, 15, 10, 9, 9, 5	—	0 in 3	0	3	33.3	
III	V +	10	All +	550.0	440.0	110.0	—	110.0	7	70.0	74	7.4	10.6	21, 17, 16, 9, 7	3, 1	0 in 3	0	5	50.0	
	V -	9	All +	333.3	222.2	111.1	—	111.1	6	66.6	62	6.9	10.3	22, * 20, 14	3, 2, 1	0 in 3	1	5	55.5	
IV	V +	10	All +	220.0	0	—	—	220.0	4	40.0	26	2.6	6.5	12, 8	4, 2	0 in 6	2	6	60.0	
	V -	10	9 +	330.0	220.0	110.0	—	110.0	8	80.0	63	6.3	7.9	19, * 14, 12	7, 5, 4, 1, 1	0 in 2	2	5	50.0	
V	V +	11	All +	436.4	327.3	1	9.1	9.1	9	81.9	74	6.7	8.2	26, 19, 14, 6†	4, 2, 1, 1, 1	0 in 2	0	7	63.6	
	V -	10	9 +	110.0	110.0	0	—	—	6	60.0	41	4.1	6.8	25	4, 4, 3, 3, 2	0 in 4	1	8	80.0	
VI	V +	10	All +	440.0	440.0	0	—	—	6	60.0	101	10.1	16.8	32, 30, 16, 12	9, 2	0 in 4	1	5	50.0	
	V -	10	All +	110.0	110.0	0	—	—	4	40.0	32	3.2	8.0	21	8, 2, 1	0 in 6	1	8	80.0	
Total	V +	60	All +	2135.0	1525.0	610.0	0.35	58.3	382	6.48	10.92						4	35	58.3	
	V -	57	55 +	2035.1	1017.6	1017.6	39	68.4	329	5.78	8.44						6	31	54.4	

TABLE I.—*Concluded.*  
*Comparison of Virus-Bearing and Virus-Free Tumor in Virus III Immune Rabbits.*

Experiment	Group	No of rabbits	Primary tumors	Mortality						Metastases				No. of metastatic foci		Probable deaths		Actual and probable recoveries
				Total		Malignant cases		Accidental deaths		Incid- ence	Total foci	Relative rate	Actual rate					
				No.	Per cent	No.	Per cent	No.	Per cent									
				No.	Per cent	No.	Per cent	No.	Per cent									
VII	V +	10	All +	2	20.0	1	10.0	6	60.0	4.0	6.7	25, 6	4, 2, 2, 1	0 in 4	0	8	80.0	
	V -	11	All +	0	—	0	—	6	55.5	1.8	3.3	—	7, 6, 3, 2, 1, 1	0 in 5	0	11	100.0	
Total of all experiments	V +	70	All +	23	32.9	16	22.9	7	10.0	41	55.9	422	6.2	10.3	4	43	61.4	
	V -	68	66 +	20	29.4	10	14.7	10	14.7	45	66.2	349	5.1	7.8	6	42	61.8	

V + = virus-bearing tumor; V - = virus-free tumor.

\* Number of metastatic foci estimated.

† Complicating empyema.



TABLE II.

*Analysis of Metastatic Foci in All Fatal Cases.*

Group	4th wk.		5th wk.		6th wk.	7th wk.		8th wk.			
Virus-bearing tumor	II	30*	II	15*		I	7	I	10		
	II	17*									
	II	15*									
			III	21*		III	17*				
			III	16*		III	9*				
						III	7				
	IV	12									
	IV	8				V	14*				
	V	26*	V	19*	V	6	VI	12*			
	VI	30*	VI	32*							
		VI	16*								
		VII	6	VII	25*						
Virus-free tumor	I	5	I	3	I	7	I	9	I	11*	
					I	15*†					
	II	9	II	22*	II	15'	II	9			
	II	5	II	10							
	III	14	III	22*†							
			III	20*	IV	12	IV	19*†		14*	
			V	25*							
	VI	21*									
Deaths 4th and 5th wks.	Malignant cases		Total foci	Foci in malignant cases		Deaths 6th, 7th and 8th wks.	Malignant cases		Total foci	Foci in malignant cases	
	No.	Per cent		No.	Per cent		No.	Per cent		No.	Per cent
V + 14	11	78.6	263	237	90.1	V + 9	5	55.5	107	77	72.0
V - 11	5	45.5	156	110	70.5	V - 9	5	55.5	111	74	66.8
Total deaths	Malignant cases			Total foci	Foci in malignant cases			Total foci	Foci in malignant cases		
	No.		Per cent		No.		Per cent		No.		Per cent
V + 23	16		69.6	370	314		84.9				
V - 20	10		50.0	267	173		64.8				

Roman numerals refer to number of experiment; Arabic to number of metastatic foci.

\* Instances of well marked malignancy.

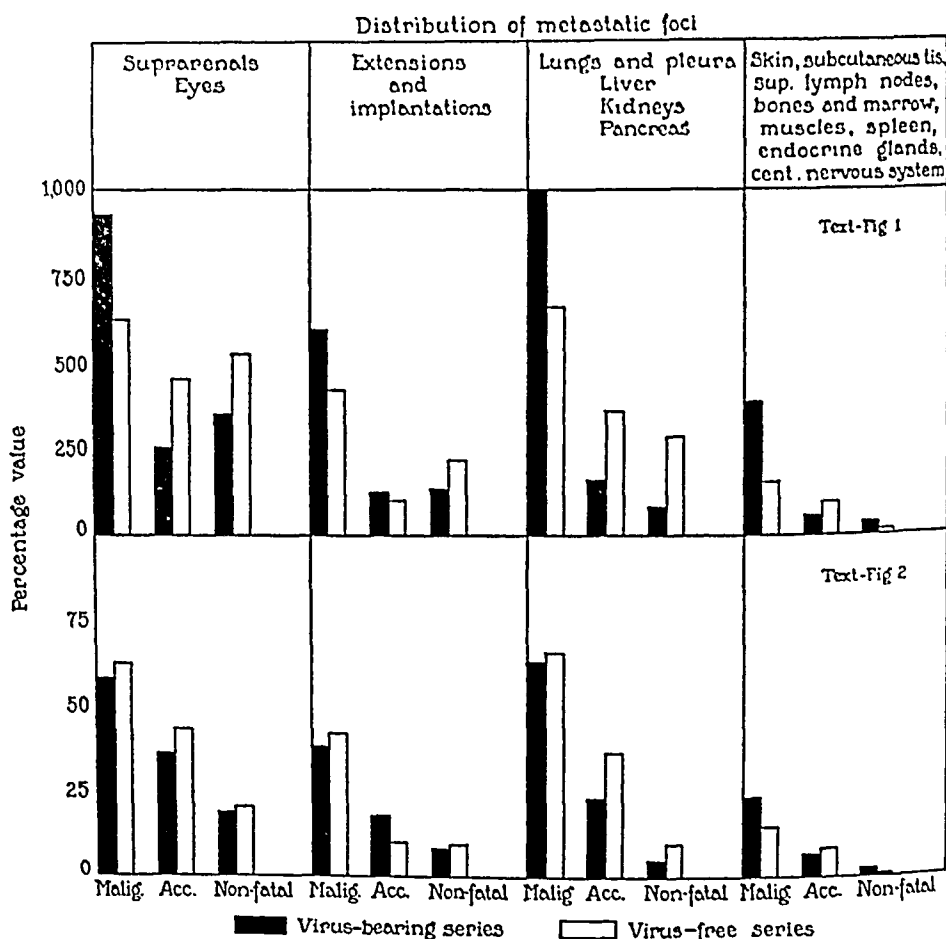
† Number of foci estimated.

TABLE III.  
*Distribution of Metastatic Foci.*

Group	No. of rabbits	I. Suprarenals and eyes				II. Extensions and implantations				III. Lungs				IV. Skin, subcut. tissue, super. lymph nodes, etc.				Total foci		
		No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No. of foci	Per cent	Foci per rabbit	Per cent	No.	Per rabbit	
Malignant cases	V +	16	37	925.0	2.31	57.8	112	593.6	7.0	37.1	50	1000.0	3.12	62.5	115	379.5	7.2	23.7	314	19.6
	V -	10	25	625.0	2.5	62.5	79	418.7	7.9	41.9	33	660.0	3.3	66.0	47	155.1	4.7	15.5	181	18.4
Accidental deaths	V +	7	10	250.0	1.43	35.7	23	121.9	3.3	17.4	8	160.0	1.42	22.9	15	50.0	2.1	7.1	56	8.0
	V -	10	17	425.0	1.7	42.5	20	106.0	2.0	10.6	18	360.0	1.8	36.0	28	92.4	2.8	9.2	83	8.3
Non-fatal cases	V +	18	14	350.0	0.78	19.4	25	132.5	1.4	7.4	4	80.0	0.2	4.4	9	29.7	0.5	1.7	52	2.9
	V -	25	21	525.0	0.84	21.0	41	217.3	1.6	8.7	14	280.0	0.6	11.2	6	19.8	0.2	0.8	82	3.3
Total	V +	41	61	1525.0	1.48	37.2	160	848.0	3.9	20.7	62	1240.0	1.5	30.2	139	458.7	3.4	11.2	422	10.3
	V -	45	63	1575.0	1.4	35.0	140	742.0	3.1	16.5	65	1300.0	1.4	28.9	81	267.3	1.8	5.9	319	7.8

*Virus-Free Strain of the Neoplasm.*—This has been described in an earlier report (4) and in the preceding paper (1).

The experiments reported in this paper were carried out in the same manner as those previously reported (1).

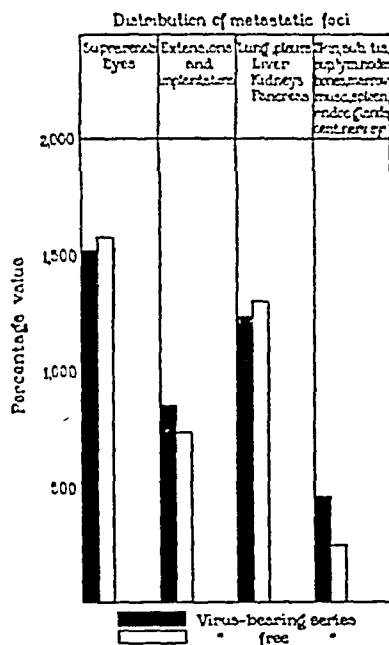


TEXT-FIG. 1. Distribution of metastatic foci in different types of disease.

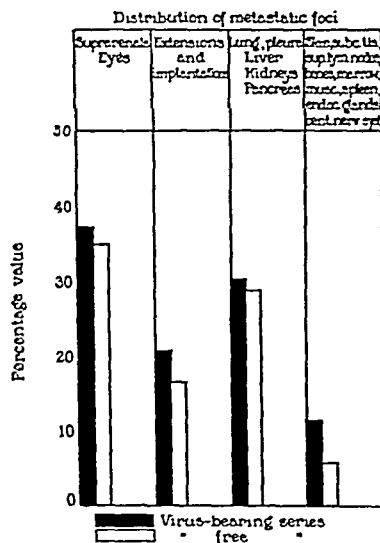
TEXT-FIG. 2. Average distribution per animal of metastatic foci in different types of disease.

*Method of Analysis of Results.*—Clinical and postmortem observations have been analyzed in the same way as those given in the preceding paper (1). The secondary growths have also been considered in relation to the organs and tissues involved.

The percentage estimations of metastatic distribution have been based upon the number of foci theoretically possible as shown by the actual location of metas-



TEXT-FIG. 3. Distribution of metastatic foci in all cases.



TEXT-FIG. 4. Average distribution per animal of metastatic foci in all cases.

tases in the first 20 generations of tumor animals (5). Certain obvious objections may be made to this method. Such organs as the liver or kidneys, for example, may show numerous tumors or only a few which destroy little of the organ. But, the general character of the disease, whether of high, moderate or low malignancy, is shown by grouping the metastatic foci in the following divisions:

I. Suprarenals and eyes.....	4 possible foci		
II. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum.....	19	"	"
III. Lungs and pleura, liver, kidneys and pancreas....	5	"	"
IV. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system.....	30	"	"

This arrangement which has been employed in analyzing the results of other tumor experiments (6) was selected upon the basis of observations of several hundred rabbits inoculated with this tumor. Suffice it to say that in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation, there is usually a widespread distribution of secondary growths to many organs and tissues, including the skin, muscles, bones and bone marrow and the endocrine glands which are practically never involved in cases of low malignancy. In those animals in which the disease is very mild, metastases may be found only in the eyes or suprarenal glands or in the facial and jaw bones and cranial sinuses which also, of course, are frequently involved in instances of a severe disease. All gradations are found between these extremes of malignancy.

### *Results.*

The results of the 7 experiments here reported consist of the clinical and postmortem observations of 138 rabbits, 70 of which were inoculated with the virus-bearing and 68 with the virus-free strain of the tumor. For convenience of discussion, these observations are summarized in Table I.

An analysis of the metastatic foci found in all fatal cases is based upon the time at which such deaths occurred with reference to the time of inoculation (Table II).

A further analysis of the character of the disease takes into account the various organs and tissues actually involved by metastases (Table III). The charts of Text-figs. 1 to 4 are graphic representations of the numerical values of Table III.

## DISCUSSION.

The experiments reported were undertaken for the purpose of ascertaining whether the presence of Virus III in a transplantable neoplasm of the rabbit influenced the character of the malignant disease. Because of the variability of the tumor process which may be influenced by many factors, the value that can be attached to differences in disease manifestations must be interpreted with due consideration of the circumstances of the experiment. In the present study the factors which could be controlled were common to both animal groups of each experiment.

The most important variable which could not be controlled was the inoculating material itself which, of course, was derived from different sources and there was no means of ascertaining whether there were actual or potential differences of growth capacity in the 2 tissues. It might be said that this difference in material constitutes an objection to the comparison of results, but since the experiments necessitated the presence of the virus in one strain of the tumor and its absence in the other, the use of transplants from different sources was unavoidable. In both strains the tumor employed for transfer was carefully selected with the intention of providing as favorable material in the one case as in the other. With the virus-bearing strain (the stock tumor) transfers were regularly made at monthly intervals as had been our custom for several years. This method was also followed with the virus-free strain during the time of these experiments, but immediately preceding the first experiment, transfers had been made more frequently which may have influenced the results obtained. The material used in the last 3 experiments was obtained from rabbits which had been immunized to Virus III, and as has been pointed out in the preceding paper (1), there is some evidence to show that material from such a source is not as favorable as that obtained from normal rabbits. This feature will subsequently be discussed more fully, but it may be stated now that material subjected to such supposedly deleterious influences as freezing, thawing and grinding in the frozen state before being used for inoculation appeared to have no appreciable effect on the character of the disease produced (7). While these procedures were extraneous as contrasted with possible effects of the virus

within the tumor, both being subjected to the influence of the animal organism, the results of the experiments referred to show that chance inequalities of material used for inoculation may not be a factor of any considerable importance where extreme variations of malignancy are concerned.

The first impression obtained from the summary of the clinical and postmortem observations in Table I is the variability of the disease picture in both the virus-bearing and the virus-free groups. This feature was, with 2 exceptions, more pronounced in the case of the virus-bearing strain, as shown by percentage variation values of various disease manifestations. In the case of the total mortality rate, the virus-free series was the more variable while the percentage variation of metastatic incidence was the same in both series.

Virus-bearing tumor						Virus-free tumor										
Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate	Total mortality	Malignant cases	Incidence of metastases	Foci of metastases	Relative rate	Actual rate					
per cent	per cent	per cent		per cent	per cent	per cent	per cent	per cent		per cent	per cent					
50.0	44.4	81.9	101	10.1	16.8	66.6	22.2	100.0	70	7.7	11.7					
44.4	40.0	70.0	81	9.0	16.2	66.3	22.2	80.0	63	6.9	10.3					
40.0	40.0	60.0	74	7.4	8.2	33.3	22.2	66.6	62	6.8	8.0					
36.0	27.3	60.0	74	6.7	6.9	30.0	20.0	66.6	61	6.3	7.8					
20.0	10.0	55.5	40	4.0	6.7	10.0	10.0	60.0	41	4.1	6.8					
20.0	0	40.0	26	2.6	6.5	10.0	10.0	55.5	32	3.2	6.8					
20.0	0	40.0	26	2.6	6.5	0	0	40.0	20	1.8	3.3					
Percentage variation.....33.7						73.3	19.2	42.1	42.2	36.3	68.1	48.0	19.7	32.4	36.3	24.4

If the results of all experiments are combined, the disease produced by the virus-bearing strain appears to be more severe (Table I). The number of cases of well marked malignancy was distinctly greater, 22.9 as contrasted with 14.7 per cent; there were more foci of metastases, 422 as compared with 349, and in addition, the relative and actual metastatic rates were higher, 6.2 and 10.3 as contrasted with 5.1 and 7.8 respectively. The incidence of metastases, however, was slightly lower, 55.9 as contrasted with 66.2 per cent. There was no

difference in the proportions of actual and probable recoveries, 61.0 per cent of each series falling in this category and little difference in the small numbers of probable deaths, so that the observations of the fatal cases are of particular importance.

In the analysis which has been made of the fatal cases (Table II), special attention is given to the time of death since the majority of rabbits with a process of pronounced malignancy die within 3 to 5 weeks after inoculation. Reference to Table II shows that approximately the same proportion of deaths in each group occurred within the 4th and 5th weeks after inoculation, that is, in the case of the virus-bearing series 14 out of 23 cases or 60.9 per cent and 11 out of 20 or 55.0 per cent in the virus-free series. But there was a decided difference in the incidence of cases of well marked malignancy, for 11 of the 14 deaths (78.6 per cent) in the virus-bearing series were of this type as contrasted with 5 of the 11 deaths (45.5 per cent) in the virus-free group. The proportion of metastatic foci in these malignant cases to the total number of foci found in all fatal cases of the 4th and 5th weeks shows a striking difference, that is, virus-bearing, 90.1 per cent; virus-free, 70.5 per cent. When the deaths which occurred in the last 3 weeks of the observation period are similarly considered, however, there is little difference between the 2 groups.

If these comparisons be made with all deaths irrespective of time, the values obtained as given below would indicate that the more severe condition developed in the disease produced by the virus-bearing tumor.

Group	Total deaths	Malignant cases		Total metastatic foci	Foci in malignant cases	
		No.	Per cent		No.	Per cent
Virus-bearing.....	23	16	69.6	370	314	84.9
Virus-free.....	20	10	50.0	267	173	64.8

The higher incidence of cases of well marked malignancy in the virus-bearing series is of considerable significance in view of the total number of animals comprising each group (Table I). There was practically no difference in the total mortality rates of the 2 series, however, nor in the time distribution of all deaths, but there was a considerable



difference in the incidence and time of the so called accidental deaths as shown by the following data:

Group	No. of rabbits	Total deaths		Accidental deaths		Deaths 4th and 5th wks.		
		No.	Per cent	No.	Per cent	Total	Accidental	
							No.	Per cent
Virus-bearing.....	70	23	32.9	7	30.4	14	3	21.4
Virus-free.....	68	20	29.4	10	50.0	11	6	54.5

The accidental deaths are directly caused by the particular location of a metastasis such as the spine, and other secondary growths are not necessarily numerous or destructive, especially if death occurs within the first few weeks. These cases cannot be dismissed as entirely lacking in malignant potentialities, however, for it is not impossible that at least some of them would have subsequently died from a more extensive tumor process had the immediate cause of an early death not occurred. But, as far as the average numerical distribution of foci in the different types of fatal cases is concerned, the following figures show little difference between the virus-bearing and the virus-free series:

*Deaths Due to Well Marked Malignancy.*

Group	4th and 5th wks.			6th, 7th and 8th wks.		
	No. of rabbits	No. of metastatic foci	Rate	No. of rabbits	No. of metastatic foci	Rate
			per cent			per cent
Virus-bearing.....	11	237	21.5	5	77	15.4
Virus-free.....	5	110	22.0	5	74	14.8

*Accidental Deaths.*

Virus-bearing.....	3	26	8.7	4	30	7.5
Virus-free.....	6	46	7.7	4	37	9.3

As far as the analysis of results has been carried, the chief points of difference between the 2 series of rabbits are (1) the higher incidence

of fatal cases with a tumor process severe enough to be classified as "malignant" in the virus-bearing series, and (2) a lower incidence of "malignant" cases but a higher incidence of early "accidental" deaths in the virus-free series.

The question at once arises as to how much importance should be attached to such points of difference as indicating variations of disease severity which could be ascribed, either directly or indirectly, to the presence or absence of Virus III in the tumor. Before this subject is discussed, the character of the disease should be considered from the standpoint of the organs and tissues involved. In such an analysis it is convenient to adopt some such plan as was outlined in the section on Materials and Method which consists in grouping the secondary tumors observed at postmortem examination into major divisions corresponding to the distribution of metastases which have been found in association with tumor processes of various degrees of severity (Table III).

The first column of each of the 4 divisions of Table III contains the total number of foci found while the figures in the second column represent the average number of foci per animal. The percentage values have been calculated upon the basis of the total number of theoretically possible growths in these sites. Text-figs. 1 to 4 are graphic representations of the values of Table III. The analysis includes all cases in which secondary growths were found and the classification of these cases previously employed has been followed, that is, malignant cases, accidental deaths and animals surviving the observation period.

Reference to Table III and to Text-fig. 1 shows that as far as the malignant cases are concerned, there were many more metastatic foci in all 4 divisions of secondary growths in the virus-bearing than in the virus-free series, which is to be expected in view of the larger number of these cases in this group. When the average value per animal is considered, however, there is little difference between the 2 series except in one important division (IV), namely, that which includes the skin, muscles, bones and endocrine glands (Text-fig. 2). Metastases to these tissues and organs rarely occur except in cases of well marked malignancy. It must be remembered, moreover, that the number of theoretically possible foci in this division is much larger than in the others, so that small differences are significant, as in the present in-

stance in which the values for the virus-bearing and virus-free series were 7.2 and 4.7 per rabbit, respectively. The distribution of secondary growths would indicate, therefore, that the tumor process in the cases of well marked malignancy was more severe in the virus-bearing than in the virus-free series.

In the group of accidental deaths, on the other hand, the disease was slightly but definitely more severe in the virus-free group (Table III; Text-figs. 1 and 2). The values for this group in the 1st, 3rd and 4th divisions of metastatic foci are all larger than those of the virus-bearing series. In the 2nd division, that of extensions and implantations, the figures for the virus-bearing series are the larger, but as has been pointed out, tumors in this category as well as those in the suprarenal glands and the eyes (Division I) possess far less significance from the standpoint of disease severity than do those in such locations as the lungs, liver, kidneys, skin, muscles, bones and other glands of internal secretion (Divisions III and IV). The reason for the higher incidence of accidental deaths in the virus-free series is not clear, but the fact that the disease in these cases was more severe in the virus-free than in the virus-bearing group suggests that under other circumstances, such as a larger number of animals or another experimental period, the malignant level of the entire virus-free series might more nearly approach that of the virus-bearing series.

If the figures for these disease types—the malignant cases, the accidental deaths and the non-fatal cases—are combined and analyzed in the same manner (Table III; Text-figs. 3 and 4), it is seen that there was practically no difference between the virus-bearing and the virus-free groups in regard to extensions and implantations (Division II), and to the distribution of metastases to the suprarenals and eyes (Division I), and to the lungs, liver and kidneys (Division III). On the other hand, the more frequent involvement of the skin, subcutaneous tissues, muscles, bones, heart, central nervous system and glands of internal secretion (Division IV) in the virus-bearing series points to a tumor process of a somewhat higher malignancy than that of the virus-free series. The conclusion from these analyses is in accord with those previously arrived at from a consideration of such features of the disease as the mortality rate, the type of fatal case and the number and rate of metastatic foci.

The combined observations of all experiments have so far been considered but if the results of individual experiments are compared, it is seen that they are not entirely constant (Table I). The degree of malignancy in Experiments II, III, V, VI and VII was greater in the virus-bearing groups as shown by the incidence of malignant cases, the total number of metastatic foci and the relative and actual rates of the growths. But in Experiments I and IV, the disease of the virus-free groups was more severe. In these 2 experiments the tumor process of the virus-bearing series was very mild, much more so, in fact, than in any other group in any experiment except the last, No. VII, in which the disease of both groups was benign. Rabbits immunized to Virus III were employed in this experiment, which condition is associated with a disease of low malignancy (1).\*

Pronounced variations in malignancy in groups of rabbits inoculated with the stock tumor at consecutive monthly intervals have been observed over long periods. From an analysis of the first 20 generations of the tumor, it appeared that the principal factors concerned in determining the results of transplantation were adaptation to passage and variations in meteorological conditions that prevailed during the time the experiments were carried out,—the one affecting the energy of cell growth, and the other affecting animal economy (7). It must be assumed that meteorological influences would be exerted over both groups of animals in each of the present experiments. On the other hand, the possibility that such an agent as Virus III might affect the energy of cell growth should be considered. The filterable viruses are intimately associated with cells, and frequently with young cells in particular, and it is possible that with the tumor some biological effect would be induced by the presence of Virus III, perhaps of the nature of a stimulation to cell growth. In the case of vaccine virus and Virus III, for example, the idea of cell stimulation, probably in connection with cell injury, is supported by the histological picture of early lesions of the cornea and skin of rabbits.

\* In Experiment VII, the disease was not as mild in the virus-bearing as in the virus-free group. It is of interest to note that of the 20 metastatic foci in the virus-free group (Table I), 1 was healed and 13 were largely or wholly necrotic. In the virus-bearing group, on the other hand, a similar condition was noted in but 3 of the 40 metastatic foci.

The growth capacity of the tumor cells may be so great that the stimulation to cell growth induced by an agent like Virus III would be entirely negligible. On the other hand, if such an influence is especially exerted upon young cells, and if it results in an increase in the rate of cell multiplication, a more vigorous growth of the early primary tumor would probably take place, which might lead to the production of a more severe disease. However, the tumor process is affected by a variety of factors and conditions, some of which might favor and others oppose the hypothetical influence of Virus III and the mildness of the disease in Experiments I and IV may be examples of the successful opposition, from an unknown cause, of the more usual effect associated with the virus.

Attention should also be called to the fact that although the disease of the virus-free groups was more uniform than that of the virus-bearing groups with respect to certain pathological manifestations, there was a definite tendency toward a lower plane of malignancy in the last as compared with the first experiments. During the course of this study, both strains of the tumor were transferred at monthly intervals and as presumably favorable material was used in the one case as in the other. But immediately preceding the experiments, the virus-free strain had been transferred more frequently, and although no conclusive evidence is available that such a procedure promotes increased malignancy, other factors being equal, still the possibility should be considered. During the latter part of this work, the virus-free tumor was carried in rabbits which had been immunized to Virus III, and observations show that in them there is a definite tendency toward a disease of lowered malignancy (1). It is possible, therefore, that the inoculating material derived from Virus III immune animals was of a less favorable character and as such may have contributed to the decreased severity of the disease as it appeared in the virus-free groups of the last 3 experiments.

Among other factors which may have indirectly influenced the course of the process induced by the virus-bearing tumor, there is one in particular which merits special attention, namely, the effect of Virus III on the rabbit host. It will be recalled that a certain number of normal rabbits, 15.0 to 20.0 per cent on an average in this laboratory, have been found to be immune to Virus III. It has also been observed

that the tumor process tends to be less severe in rabbits which have been experimentally immunized to Virus III than in normal rabbits (1). From these facts, it would appear that the chance inclusion of one or more Virus III immune rabbits in a group of 10 animals would affect the experimental results in the direction of diminished malignancy. There was just as much chance, however, for immune rabbits to be included in the virus-bearing as in the virus-free groups. All rabbits inoculated with the virus-free tumor which were subsequently tested for an immunity to Virus III showed a typical cutaneous reaction, indicating the absence of an immunity. Preliminary virucidal tests of the sera of rabbits subsequently inoculated with the virus-bearing tumor were not carried out in the present series of experiments, so that it is impossible to say whether any Virus III immune animals were included in these groups.

On the other hand, one must take into account the possible effect of Virus III upon the course of the disease from the standpoint of the more immediate reaction of the animal host to the introduction of the virus with the tumor. At first glance, one might suppose that the severity of the tumor process would be diminished since the general plane of malignancy tends to be low in rabbits immunized to Virus III and rabbits inoculated with the virus-bearing tumor develop an immunity to the virus. It has been shown, however, that such is not the case, the probable explanation being that the immunity occasioned by the virus-bearing tumor develops more slowly than that intentionally caused by the injection of tissue emulsions rich in virus content (1). When an immunity is fully developed in connection with virus-bearing transplants, the tumor process has apparently reached the point when it is not affected by the immune state of the host, to the degree, at least, that is observed in rabbits which are already immune to Virus III at the time of tumor inoculation. On the other hand, the presence of the virus within the tumor affects the animal host since an immunity to it eventually develops, and it is not unlikely that during the development of this state, the response of the host to other pathogenic agents would be affected. Thus, in the present instance, the somewhat greater severity of the disease induced by the virus-bearing strain may be due to an increased susceptibility or a decreased resistance of the host as a collateral or indirect result of the

Virus III infection of the tumor transplant. In Experiments I and IV, in which the disease was unusually mild, it is not unreasonable to assume that there were present factors or conditions which operated to prevent this alteration in the reaction of the host to the tumor or which effectively opposed the result of such an alteration. An unusually low content or diminished activity of Virus III in the material used for inoculation or an already existing immunity to Virus III are factors which might operate in this manner.

The reciprocal effects of concomitant or superimposed disease conditions have received considerable attention, chiefly, however, from a clinical standpoint. That the subject is open to experimental investigation is shown by the results of the present study which suggest that the presence of one pathogenic agent, a filterable virus, is associated with certain variations in the disease induced by a second agent, the malignant tumor. That these variations are not of larger magnitude may be related to the virulence of the virus which is not high as ordinarily considered in terms of the usual criteria of animal reaction, so that a pronounced effect would hardly be expected.

In conclusion, it should be pointed out that the influence of an associated infection with Virus III as a factor concerned in determining variations in growth and malignancy of the tumor must be evaluated with due regard to the many other factors which influence the disease process. There is no indication, for instance, that extreme variations in malignancy could be accounted for on this basis, or that the orderly succession of periods of increasing and diminishing malignancy with a distinct and characteristic tendency to a seasonal distribution are affected by the presence or absence of Virus III in the tumor. The present observations, however, indicate that the presence of Virus III is usually associated with a higher degree of malignancy while the effect of an immunity to Virus III has been shown to be associated with a disease of diminished severity (1). These conditions probably account for some of the irregularities in results observed in individual animals and in certain groups of animals.

#### SUMMARY AND CONCLUSIONS.

A study of a malignant disease in rabbits has been made with reference to the presence or absence of a filterable virus, Virus III, in the

tumor. The results are analyzed from the standpoint of certain characteristic features of the tumor process in order to determine any differences in degrees of malignancy.

It was found that a more severe disease developed in the series in which the virus-bearing tumor was used than in the series in which the tumor was free of the virus, although the differences were not very marked and were not entirely constant.

The influence of Virus III as a factor affecting malignancy has been discussed from the standpoint of its possible effect upon (a) the tumor cells and (b) the host reaction. It has been suggested that the greater malignancy of the pathological process usually induced by the virus-bearing tumor is attributable to a change in the response of the host to the tumor, which change is of the nature of a decreased resistance associated with the reaction of the host to the virus infection.

#### BIBLIOGRAPHY.

1. Pearce, L., and Rivers, T. M., *J. Exp. Med.*, 1927, xli, 65.
2. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 601.  
Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxvii, 631.  
Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 799.  
Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxvii, 811; x, xviii, 347, 367.  
Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxviii, 385.
3. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 583.  
Pearce, L., Brown, W. H., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 603.
3. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673; 1924, xxxix, 777; xl, 281.
4. Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, xlii, 523.
5. Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxviii, 347.
6. Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 431; 1927, xlv, 483.  
Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1927, xlv, 727.
7. Pearce, L., Brown, W. H., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 603.





# IMMUNOLOGICAL PROPERTIES OF A TYPICAL (S-PRODUCING) AND A DEGRADED (NON-S-PRODUCING) STRAIN OF TYPE II PNEUMOCOCCUS WITH SPECIAL REFERENCE TO PROTECTIVE ANTIBODIES.

BY EMIDIO L. GASPARI,\* WILLIAM L. FLEMING, AND  
JAMES M. NEILL, PH.D.

*(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)*

(Received for publication, March 28, 1927.)

## INTRODUCTION.

The advance in the knowledge of the chemical nature of the carbohydrate substance (1-3) of *Pneumococcus* makes it desirable to obtain further evidence of its biological significance. Facts of interest in this connection have been furnished by the reports (4) of the changes in immunological properties exhibited by pneumococci which have lost the specialized cellular function of elaborating the soluble specific substance (carbohydrate). Reimann's (5) recent paper adds value to the earlier work (4), since his results indicate that the immunological differences accompanying the loss of the S-producing function can be explained upon the basis of Avery and Heidelberger's interpretation of the immunological relations of pneumococcus cell constituents.

It is the object of the present paper to present evidence of similar nature, but with special emphasis upon the immunological reactions involved in the protection of mice against virulent Type II pneumococci. The study consisted of a comparison of the immunological properties of two strains of pneumococci: a type-specific strain in which the S-producing function of the cell was highly developed; and a non-type-specific strain in which the S-producing function was completely lost. The comparison of the two strains from the stand-

\* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

point of antipneumococcus protection of mice was investigated by active and passive immunity tests, and by the absorption of anti-pneumococcus serum with whole bacterial cells and with culture filtrates.

#### EXPERIMENTAL.

*Strains of Pneumococci.*—Two strains of pneumococci were employed in the investigation: (1) an S-producing<sup>1</sup> (Type II) strain; (2) a non-S-producing<sup>1</sup> strain derived by the "spontaneous degradation" of the first strain. *S-Producing Strain.*—Strain D<sub>39</sub>, Type II (Hospital of The Rockefeller Institute) was used as the S-producing or type-specific strain. It was highly virulent, its lethal dose for mice never being greater than one-millionth cc. of culture. *Non-S-Producing Strain.*—The non-S-producing or non-type-specific strain was derived from the above type-specific strain by repeated transfers in broth made at infrequent intervals. The culture finally used had passed through 50 successive transfers in broth at intervals of 2 to 3 days; it had completely lost its S-producing function, and was no longer pathogenic for mice.

#### *Serological Reactions of the S-Producing and Non-S-Producing Strains of Pneumococci.*

In type-specific antipneumococcus serum, the S-producing strain exhibited the usual serological reactions; the bacteria themselves were agglutinated and the carbohydrate or S substance in culture filtrates was precipitated. In contrast, the bacterial cells of the non-S-producing strain were not agglutinated, nor were the supernatants of its broth cultures precipitated. The results obtained in species-specific antipneumococcus protein serum were quite different. The S-producing strain was not specifically agglutinated, nor was the carbohydrate substance in its filtrates precipitated. The non-S-producing strain, on the other hand, was agglutinated by the serum which contained the species-specific antiprotein antibodies.

The differences between the serological reactions of the S-producing and non-S-producing strains are essentially the same as those which have been reported by Reimann. The differences in agglutinability can be explained upon the basis of Avery and Heidelberger's schematic interpretation of the structure of the pneumococcus cell. The type-specific agglutination of the S-producing strain may be

---

<sup>1</sup> The term "S-producing" strain as employed throughout this paper refers to the original, virulent, highly type-specific strain which possesses the specialized cellular function of elaborating the immunologically reactive carbohydrate substance. The same properties are exhibited by Reimann's "S" strains.

The term "non-S-producing" strain refers to the avirulent, non-type-specific strain which has lost the property of producing the soluble carbohydrate. Its properties are similar to those of Reimann's "degraded" or "R" strains.

referred to the reaction between the ectoplasmic layer of specific carbohydrate and the type-specific anticarbohydrate antibodies; the species-specific agglutination of the non-S-producing strain may be referred to the reaction between the protein on the outside layer of the degraded bacterial cell and the species-specific antiprotein antibodies.

### *Antigenic Properties of the S-Producing and Non-S-Producing Strains of Pneumococci.*

The differences in the antibody-invoking properties of the S-producing and non-S-producing strains were investigated by immunizing two series of rabbits with heated suspensions of the two sorts of pneumococci. The production of test-tube demonstrable antibodies was essentially the same as that reported by Reimann (5). The S-producing strain invoked the production of anticarbohydrate antibodies (type-specific agglutinins and type-specific S precipitins). The non-S-producing strain, on the other hand, was entirely devoid of the capacity to invoke the anticarbohydrate antibodies, and the serum obtained contained only the species-specific antiprotein antibodies and none of the type-specific antibodies.

The differences in the protective value of the serum yielded by immunization with the two strains are of more importance in the present paper than are the above serological reactions. The serum of animals immunized with the S-producing strain had a high passive protective value, regularly protecting mice against 0.01 cc. of virulent Type II pneumococci. In contrast, the serum of all animals immunized with the non-S-producing strain was entirely devoid of any protective power, although it possessed a high titre of species-specific antiprotein antibodies. Apparently, when the Type II pneumococcus cell loses its S-producing function, it no longer possesses the antigenic complex which invokes the production of passively protecting antibodies.

### *Active Immunization of Mice with S-Producing and Non-S-Producing Pneumococci.*

Since there are frequent differences between passive and active antibacterial immunity (8), the following experiments were made to determine whether or not active immunity could be established by the vaccination of mice with non-S-producing pneumococci.

One series of ten mice was immunized with heat-killed vaccine prepared from the type-specific or S-producing strain, and another series with vaccine from the non-S-producing strain of pneumococci. Three courses of subcutaneous injec-

tions were given with a rest period of 1 week between courses; each course consisted of five doses of vaccine, each dose being given at 2 day intervals, equivalent to 0.15 cc. of broth culture. Tests for active immunity were made 11 days after the last vaccination, by the intraperitoneal injection of different amounts of Type II pneumococcus culture ( $10^{-6}$  to  $10^{-3}$  cc.). Normal mice (not vaccinated) were injected with  $10^{-6}$  cc. as virulence controls of the culture.

The results of the active immunity tests revealed the same differences in the antigenic properties of the two strains as those previously observed in the passive protection experiments. Although the protection was not uniform, some of the mice which had been actively immunized with the S-producing pneumococci survived the injection of doses of virulent Type II organisms equivalent to as much as one-thousandth cc. of culture.<sup>2</sup> In contrast to the series immunized with the type-specific organisms, none of the animals which had been vaccinated with the non-S-producing strain had acquired any detectable immunity and all were infected by doses of one-millionth cc. of the virulent culture. Apparently, therefore, the loss of the S-producing function of Type II pneumococci was accompanied by loss of the capacity to establish active immunity in mice, as well as by loss of the capacity to invoke passively protecting antibodies.

*Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococcus Cells.*

The results of preceding experiments have shown distinct differences in the antibody-invoking properties of the S-producing and non-S-producing pneumococci: the serum of animals immunized with the S-producing strain contained type-specific antibodies (agglutinins and S precipitins) and passively protected mice against Type II pneumococcus infection; in contrast, the serum of animals immunized with the non-S-producing strain contained no demonstrable type-specific anti-

<sup>2</sup> The irregularity in the protection of the mice in this series of vaccinated mice had no relation to the dose of pneumococci injected in the active immunity tests. Some individuals injected with  $10^{-6}$  cc. and  $10^{-5}$  cc. were infected, while others injected with  $10^{-4}$  cc. and  $10^{-3}$  cc. were protected. As reported in the following paper, the results of an investigation with a larger series of mice show that the above irregularities were due to differences in the immunity response of the individual mice.

bodies and did not protect mice. In view of these marked differences in the antibody-invoking properties of the two strains, experiments were made to determine if like differences existed in the capacity of the two strains to "absorb" or remove antibodies from type-specific immune serum.

A type-specific serum obtained by immunization of a rabbit with Type II pneumococcus vaccine was used in the absorption experiment. This serum contained the usual type-specific agglutinins, S precipitins, and passively protecting antibodies.

10 cc. of serum were placed in each of three tubes. 10 cc. of a salt solution suspension of heat-killed bacteria of the S-producing strain were added to one tube of serum; the same amount of a suspension of the non-S-producing pneumococci

TABLE I.

*Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococci.*

Type-specific antipneumococcus serum	Type II agglutinins	Precipitins for Type II soluble substance	Passively protecting antibodies
Unabsorbed.....	+	+	+
Absorbed with S-producing Type II pneumococci.....	0	0	0
Absorbed with non-S-producing pneumococci.	+	+	+

was added to the second tube of serum; an equal volume of salt solution was added to the third tube. The three mixtures were shaken for 5 minutes, then incubated for 2 hours at 37°C., and finally placed in the ice box for 12 hours. The tubes were then centrifuged to remove the bacteria. 10 cc. of the supernatant fluid from the above mixtures were then placed in each of three tubes and absorbed a second time by the same procedure.

After the removal of the bacteria used in the second absorption, the three sera ((1) unabsorbed control, (2) absorbed with S-producing pneumococci, (3) absorbed with non-S-producing pneumococci) were then tested for the presence of type-specific agglutinins, type-specific S precipitins, and type-specific protective antibodies. The agglutination and protection tests were made by the usual procedure. In the S precipitin tests, the bacteria-free filtrate of a young unautolyzed culture of Type II pneumococci was substituted for a solution of the purified carbohydrate.

The results of the experiment are summarized in Table I.

The results of the absorption experiments (Table I) reveal the same general immunological differences as those obtained in the preceding

immunization experiments. Absorption with the heat-killed cells of the S-producing strain removes all the S precipitins, type-specific agglutinins, and protective antibodies, just as immunization with the bacterial cells of this strain invokes the production of these same type-specific antibodies. On the other hand, absorption with cells of the strain in which the S-producing function has been lost fails to remove any of these antibodies, just as immunization with cells of this strain failed to stimulate the production of type-specific antibodies. Hence, the pneumococcus cells which have lost their S-producing function have lost not only their property of invoking type-specific antibodies by antigenic stimulation in the animal body, but have also lost their capacity to "absorb" the same antibodies by type-specific antigen-antibody combinations in the test-tube.

*Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.*

In the following experiment, Type II antipneumococcus serum was absorbed with filtrates of young broth cultures of the S-producing and non-S-producing pneumococci. It seemed that the results to be obtained by absorption of the serum with the filtrates should be comparable to those obtained by use of solutions of the chemically purified carbohydrate since the S substance is the only immunologically reactive substance which can be demonstrated in filtrates of young and unautolyzed broth cultures of *Pneumococcus*. (The S substance is liberated into the culture fluid in the early period of growth and if the fluids are filtered before cell disintegration has commenced, the filtrates are entirely free of any of the bacterial protein or other serologically reactive substances.) To obtain fluids containing the maximum amount of the soluble substance, the cultures were planted in glucose broth. The reaction of the cultures was observed closely and when a pH of approximately 6.5 was reached, the bacteria were removed by centrifugation, the reaction adjusted to pH 7.3, and the fluids filtered through a Berkefeld candle.

5 cc. of the antipneumococcus (Type II) serum were then placed in each of three tubes. 15 cc. of the filtrate from the S-producing culture were added to the first tube; 15 cc. of the filtrate from the non-S-producing culture were added to the second tube; an equal volume of broth was added to the third tube as a control. All the mixtures were shaken for 15 minutes, incubated for 3 hours at 37°C., and then placed in the ice box for 18 hours. The mixtures were then centrifuged and the clear supernatant fluids removed. A second absorption similar to the above was carried out with 10 cc. of the supernatant fluid of each of the above serum mixtures. Only slight precipitation occurred when the S-containing filtrate was

added to the serum which had received the preceding absorption treatment with this filtrate. After 3 hours at 37°C. and 18 hours at 5°C., the second series of mixtures was centrifuged and the supernatant fluids again removed. The supernatant fluids were then subjected to a third absorption by the described procedure. The mixtures were again centrifuged and the supernatant fluids removed. The supernatant fluids of the third absorption series were employed in the following tests. The sera at this stage had been diluted to about one-twenty-fifth of their volume, so that each cc. of the test sera contained approximately 0.04 cc. of serum and 0.96 cc. of broth, or culture filtrate.

Tests for type-specific agglutinins were made by the usual method with dilutions equivalent to 1/25 and 1/100 of the original serum. Two series of passive protection tests were made with different amounts of serum. One series of mice

TABLE II.

*Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.*

Type II antipneumococcus serum	Passive protection					Type-specific agglutinins	
	Mice injected with Type II pneumococci					Serum dilutions	
	Amount of culture						
	10 <sup>-2</sup> cc.	10 <sup>-3</sup> cc.	10 <sup>-4</sup> cc.	10 <sup>-5</sup> cc.	10 <sup>-6</sup> cc.	1/25	1/100
Unabsorbed control.....	S	S	S	S	S	+	+
Absorbed with culture filtrate of S-producing pneumococci...	D	D	D	D	D	0	0
Absorbed with culture filtrate of non-S-producing pneumococci.....	S	S	S	S	S	+	+

S = survived 7 days. D = died 24 to 48 hours after injection.

received absorbed serum equivalent to 0.04 cc. of the original serum mixed with increments of broth culture ranging from 10<sup>-2</sup> cc. to 10<sup>-6</sup> cc. A second series of mice received the equivalent of 0.01 cc. of serum mixed with amounts of broth culture ranging from 10<sup>-3</sup> cc. to 10<sup>-6</sup> cc. The second series of protection tests was included to detect any incomplete absorption of protective antibodies which might not be recognized in tests with an excess of serum. Since the tests with the two amounts of serum gave identical results, only the results of the series with 0.04 cc. of serum are included in the summarized protocol presented in Table II.

Since the S substance was used for the absorption in the form of a solution in the filtrate of the S-producing strain, a slight excess of the carbohydrate remained in the serum mixture after the final absorption. Thus, it is obvious that a certain amount of the free carbohydrate was injected into the animals in the protection



tests with absorbed serum. In the absence of any evidence of an "aggrassin"-like effect of small amounts of the S substance, we do not believe that this complicated the results of the experiment.

This experiment (Table II) shows that absorption with the filtrate of the S-producing strain removed all the type-specific agglutinins and also all the protective antibodies,<sup>3</sup> while the filtrate of the non-S-producing strain failed entirely to remove any of the type-specific antibodies and did not diminish the protective value of the serum. These results of the absorption with the culture filtrates were exactly analogous to the results (Table I) of the absorption with the heated whole cells of the two strains of pneumococci and, because of the simpler nature of the culture filtrates, furnish more direct evidence of the relation of Type II anti-S antibodies to protection.

The above results are also of interest from the standpoint of the identity of the type-specific agglutinin and the anti-S precipitin. Avery and Heidelberger's (3) contention that S precipitation and type-specific agglutination involve the same antigen-antibody combination, is supported by the fact that S precipitins are removed by absorption with suspensions of washed pneumococcus cells (Table I) and conversely by the fact that the type-specific agglutinins are removed by absorption with solutions of the S substance (Table II).

The studies of the soluble substance of *Pneumococcus* by Avery and Heidelberger (2, 3) and of the similar products of other bacteria by Zinsser and Mueller (9) have presented new conceptions of the importance of carbohydrates in immunology. From this point of view, the results in Table II are of general interest as an example of the removal of the protective antibodies of an antibacterial serum by absorption with solutions in which the only reactive substance is carbohydrate in nature.

#### DISCUSSION.

The elaboration of the soluble specific substance (carbohydrate) is a specialized function of the pneumococcus cell which is most highly

<sup>3</sup> It is important to note that this complete removal of protective antibodies by absorption with the S-containing fluid occurred in Type II immune rabbit serum. In similar experiments with Type I serum from an immune horse, protective antibodies were not completely absorbed when treated with culture fluid containing the Type I reactive carbohydrate.

developed in virulent strains. The loss of the function of S production is accompanied by changes in the immunological properties of pneumococci. As previously pointed out by Reimann (5), strains of pneumococci which no longer elaborate the specific carbohydrate, lose their type-specific serological properties and exhibit antigenic properties similar to those of solutions of pneumococcus protein (6, 7). In addition to the immunological changes manifested by differences in test-tube serological reactions, the loss of the S-producing function was also accompanied by marked changes in the antigenic properties involved in both passive and active protection of mice. The injection of S-producing pneumococci stimulated the production of passively protecting antibodies in rabbits and also established active immunity in mice. On the other hand, the similar injection of non-S-producing pneumococci neither invoked the production of passively protecting antibodies nor established active immunity.

The intimate relation of the type-specific antibodies to the anti-pneumococcus protection of mice was likewise evident in the results of absorption experiments. Absorption of Type II antipneumococcus serum with the heat-killed cells of S-producing pneumococci of homologous type removed the commonly recognized antibodies concerned in type-specific agglutination and in type-specific precipitation of solutions of the S substance. Serum from which these type-specific antibodies had been removed were completely devoid of passively protecting antibodies. On the other hand, when the Type II serum was subjected to the same absorption treatment with non-S-producing pneumococci, there was no detectable loss in the type-specific antibodies responsible for agglutination and S precipitation. This absorbed serum from which the type-specific antibodies had not been removed, possessed the same protective value as unabsorbed serum. More direct evidence of the relationship between the anticarbohydrate and the protective antibodies was furnished by the fact that absorption of Type II antipneumococcus serum with culture filtrates containing the S substance in solution removed the protective antibodies as completely as did the whole bacteria themselves. It is reasonable to believe that the removal of the protective antibodies was due to the reaction between the carbohydrate S substance and its type-specific antibody.

Although antipneumococcus immunity is type-specific and consequently the antibodies that confer it must also be type-specific, it is hazardous to conclude that Type II antipneumococcus protection is simply a function of the anticarbohydrate antibody. A number of reports have been made of the protective value of antipneumococcus serum which possesses none of the usual test-tube demonstrable antibodies. However, all our results indicate that the type-specific anticarbohydrate antibody is at least the dominant one involved in the protection of mice against virulent Type II pneumococci.

It is common experience (10) to find that the production of effective antipneumococcus serum requires the use of virulent pneumococci in the immunization. If the anti-S antibody is the one most prominently involved in Type II passive protection, the failure to obtain an effectively protecting serum by immunization with avirulent pneumococci would be explained not by the loss of the virulence of the bacteria employed as antigens, but by the fact that the avirulent bacteria no longer possess the antigenic complex (*SP* of Avery and Heidelberger (3)) which is required for the immunological production of type-specific antibodies.

#### SUMMARY.

The loss of the specialized function of S production by Type II pneumococcus was accompanied by a loss of the antigenic properties involved in both active and passive protection of mice. Absorption of Type II serum with S-producing pneumococci removed all the protective antibodies, as well as the type-specific agglutinins and S precipitins. The same absorption treatment of the serum by non-S-producing pneumococci failed entirely to remove the type-specific antibodies and did not affect the protective value of the serum. Absorption with bacteria-free culture fluids containing the reactive carbohydrate removed the protective antibodies as completely as absorption with the whole bacterial cells of type-specific strains. The results taken as a whole indicate that the antibodies involved in the usual protection of mice against Type II pneumococci are closely related, if not identical, to the specific anticarbohydrate precipitin.

## BIBLIOGRAPHY.

1. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
2. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
3. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367.
4. Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49; Griffith, F., *Rep. Pub. Health and Med. Subj.*, No. 18, 1923, 1; Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587; Amoss, H. L., *J. Exp. Med.*, 1925, xli, 649.
5. Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 107.
6. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.
7. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1925, xlii, 355.
8. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.
9. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275; Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343; Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159; and other papers.
10. Neufeld, F., and Händel, Z. *Immunitätsforsch., Orig.*, 1909, iii, 159; *Arch. k. Gsndhtsamte*, 1910, xxxiv, 166, 293.



# FACTORS INVOLVED IN THE INFECTION OF MICE AFTER VACCINATION WITH TYPE II PNEUMOCOCCI.

By JAMES M. NEILL, PH.D., AND EMIDIO L. GASPARI.\*

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

(Received for publication, March 28, 1927.)

In a previous study<sup>1</sup> it was found that vaccination of a group of mice did not protect all the individual animals against subsequent infection with Type II pneumococci. The protection obtained was irregular; some individuals resisted relatively large numbers of pneumococci, while other individuals succumbed to much smaller doses. The present paper deals with a further study of the infection of individual mice after vaccination against Type II pneumococci. The experiments were planned to determine the relative importance of two factors in the postvaccination infections: the previous immunity response of the individual animals, and the dosage of the invading bacteria. The factor of differences in the immunity response of the individual is made especially prominent, for the animals (mice) not only possess no demonstrable natural immunity to the specific bacteria (pneumococci) but the vaccination was done with a type of *Pneumococcus* known not to produce a highly effective antibacterial serum.

## EXPERIMENTAL.

### *Methods.*

*Immunization.*—70 line-bred females, between 3 and 4 months old, which had been separated from males since weaning were selected for immunization.

The organisms from a 10 hour broth culture of Type II pneumococci (Strain D<sub>39</sub>, Hospital of The Rockefeller Institute) were resuspended in salt solution and heated at 60°C. for 30 minutes. Each mouse received, subcutaneously, 0.5 cc. of a

\* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

<sup>1</sup> Gaspari, E. L., Fleming, W. L., and Neill, J. M., *J. Exp. Med.*, 1927, xlv, 101.

diluted suspension of the vaccine (equivalent to 0.1 cc. of broth culture) every 2 days for six doses. 10 days after the last injection a number of mice were tested for acquired immunity. The remainder were given a second course of immunizations with freshly prepared vaccine at 2 day intervals for five doses. After a rest period of 10 days, a number of animals were tested for immunity and the remainder were given a third course of immunizations with fresh vaccine exactly as in the second course; these animals were also tested for acquired immunity 10 days after the last injection.

A number of mice died during the period of immunization, and others were eliminated from the tests because of evident poor condition.

*Tests of Immunity of the Animals (Protection Tests).*—The tests of immunity of the vaccinated animals were made by intraperitoneal injections of 0.5 cc. of broth containing the desired amount of a 10 hour broth culture of the same virulent strain as that from which the vaccine had been prepared.

*Experiments 1, 2, and 3: Influence of Size of Dose upon Infection:  
Active Immunity Tests of Vaccinated Mice by Injection of  
Different Numbers of Bacteria.*

The first series of experiments (Experiments 1, 2, and 3) consisted of tests of the influence of size of dose upon the occurrence of infections in groups of mice which had previously received vaccination against Type II pneumococci. The doses used in these active immunity tests were  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  cc. of broth culture of pneumococci; this range of doses includes the numbers of pneumococci against which passive protection is usually effective. The mice used in Experiment 1 had received one course of vaccination before the immunity tests; the animals used in Experiments 2 and 3 had received, respectively, two and three courses. The purposes of the investigation are met by presenting the three experiments collectively, since each animal had received the same immunization treatment as the other individuals used in the same experiment. The results of Experiments 1, 2, and 3 are presented in Table I; a summary of these results is collected in Table II.

*Injections and Virulence Controls.*—The vaccinated mice were injected as described under "Methods." The normal mice which were injected for virulence controls invariably succumbed to all amounts of culture tested—i.e., from  $10^{-7}$  cc. to  $10^{-3}$  cc. The results of the virulence controls are presented collectively in the last column of Table I.

*Control of Possible Effect of Weight of Individual Animals.*—All the mice used in

TABLE I.  
*Tests of the Active Immunity of Vaccinated Mice.*

Amount culture injected	Experiment 1. Immunity tests on first group of mice (after one course of vaccinations)	Experiment 2. Immunity tests on second group of mice (after two courses of vaccinations)	Experiment 3. Immunity tests on third group of mice (after three courses of vaccinations)	Virulence controls* (non-immunized mice) on Experiments 1, 2, 3
cc. $10^{-3}$	D 24-48 hrs. S 5 days	D 24-48 hrs. D 24-48 hrs. D 24-48 hrs.	D 24-48 hrs. D 24-48 hrs. D 24-48 hrs. D 24-48 hrs. D 48-72 hrs. S 5 days S 5 days	D 24-72 hrs. D 24-72 hrs.
$10^{-4}$	D 24-48 hrs. S 5 days	D 48-72 hrs. S 5 days S 5 days	D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. S 5 days	D 24-72 hrs. D 24-72 hrs.
$10^{-5}$	D 24-48 hrs. S 5 days	D 24-48 hrs. D 24-48 hrs. S 5 days	D 24-48 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. S 5 days S 5 days	D 24-72 hrs. D 24-72 hrs.
$10^{-6}$	D 24-48 hrs. S 5 days	S 5 days S 5 days S 5 days	D 24-48 hrs. D 24-48 hrs. D 24-48 hrs. D 48-72 hrs. D 48-72 hrs. D 48-72 hrs. S 5 days	D 24-72 hrs. D 24-72 hrs.
$10^{-7}$	No tests	No tests	No tests	D 24-72 hrs. D 24-72 hrs.

D = died of pneumococcus septicemia.

S = survived.

\* Presented in composite form; duplicate mice were injected with each amount of culture in each of the three experiments.



the experiments were in excellent physical condition but varied in weight from 24 to 31 gm. To rule out any possible influence of weight upon the resistance to infection, the mice of different weights in Experiments 2 and 3 were matched so that the tests of the different amounts of culture were made with a series of animals of comparable weight. The results showed that there was no correlation between weight of the animal and its susceptibility.

*Survival in Table I.*—Since all the virulence controls died in less than 72 hours after injection the protection of the vaccinated mice is based upon survival for 5 days after the test injection. Three of the mice reported as "Survived" in Table I died between 10 and 14 days after injection.

The most obvious fact in Table I is the irregularity in the survival and death of the vaccinated mice. In each of the three experiments individual mice were infected by doses smaller than the doses which

TABLE II.

*Summary of Tests for Active Immunity of All the Vaccinated Mice: Proportion of Animals Protected against Different Doses of Pneumococci.*

Amount of culture injected.	Number of vaccinated mice tested	Number of mice protected	Proportion of mice protected
cc.			per cent
$10^{-3}$	12	3	25
$10^{-4}$	12	4	33
$10^{-5}$	12	4	33
$10^{-6}$	12	5	42
$10^{-3}$ to $10^{-6}$	48	16	33

other mice survived and *vice versa*. This lack of relationship between the number of pneumococci injected and the infection of the vaccinated animals is emphasized in the summary of the experiments given in Table II. There it is seen that about the same proportion of the vaccinated mice survived the injection of each of the different doses; in fact, any deviation from the average proportion of one-third survivors represents the survival or death of only one mouse.

Since these facts indicate that within this range of dosage ( $10^{-6}$  to  $10^{-3}$  cc.) the size of the dose does not influence the infection of the vaccinated animals, the infection or resistance of individuals would logically be attributed to differences in individual immunity. In these experiments the immunity of the animals was limited to that

acquired by vaccination. Therefore, within the range of dosage used in these experiments, the previous immunity response of the individual appeared to be the factor determining the postvaccination infections.

### *Obtaining a "Selected Group" of Actively Immune Mice.*

The preceding results indicated that only a certain proportion of the mice respond effectively to pneumococcus (Type II) vaccine. This fact made it impossible to carry out additional experiments until we obtained a selected group of vaccinated mice. It seemed that a "selected group" of individuals known to possess an effective active immunity could be obtained by collecting all the mice which had survived the previous injections of live bacteria in Experiments 1, 2, and 3. These mice were given the following additional vaccination treatment.

*Survivors of Experiment 1A.*—10 days after the injection made in Experiment 1, all the surviving mice were put upon a vaccination schedule similar to that described under "Methods;" in addition to the immunization preceding the tests of Experiment 1, they received three vaccination courses of five injections each.

*Survivors of Experiment 2A.*—These mice received, in addition to the immunization preceding the tests of Experiment 2, two courses of the described vaccination.

*Survivors of Experiment 3A.*—These received one additional course of vaccinations. A few mice died during the immunization period.

These mice represent a "selected group" of animals known by previous tests to possess some degree of active immunity against Type II pneumococci and were used as described in the following experiments.

### *Occurrence of True Mass Infection in Actively Immune Mice.*

It is an established fact that no amount of antipneumococcus serum can passively protect mice against overwhelmingly large numbers of virulent pneumococci. This phenomenon can properly be termed "true" mass infection, in that the passive immunity furnished by the antibacterial serum is known to be sufficient to protect against infection by smaller numbers of the bacteria. Experiments were made to determine whether active immunity likewise fails when the vaccinated animal is invaded by doses beyond the zone within which passive protection is effective. In view of the previously demonstrated differences in the response of individual mice to vaccination, the tests for "mass infection" were limited to animals known to have acquired sufficient active immunity to resist invasion by smaller numbers of pneumococci.

Four mice from the previously described "selected group" of actively immune mice were injected with 0.02 cc. of broth culture of Type II pneumococci. Although these mice possessed a fairly high degree of antipneumococcus active immunity, they all died of pneumococcus septicemia. (That all of them possessed

at that time sufficient immunity to protect them against at least  $10^{-4}$  cc. of broth culture is shown by the fact that all other individuals of the "selected" group survived that dosage when tested in a simultaneous experiment.)

The results of this experiment showed that, even in a selected group of actively immune mice, infection invariably follows invasion by overwhelmingly large numbers of pneumococci regardless of the previous immunity response of the individual. This experiment presents a definitely controlled example of the true mass infection of actively immunized animals, in that the infected individuals were known to possess sufficient immunity to protect them against smaller numbers of pneumococci.

*Comparison of the Occurrence of Infections in an "Unselected" and in a "Selected" Group of Vaccinated Mice When All the Individuals Are Injected with the Same Number of Pneumococci (Type II).*

The importance of the factor of individual immunity response in postvaccination infection was further illustrated by the following comparison of the percentage of infections which occur when a constant number of bacteria is injected into two different groups of vaccinated mice.

In one group, the factor of differences in individual immunity response was eliminated by including only animals known by previous test to have sufficient immunity to resist the invasion of at least a small number of pneumococci; in the other group, this factor was uncontrolled (as in Experiments 1, 2, and 3), and although all the animals had received the same vaccinations, no previous tests had been made to determine the effectiveness of their immunity response. The same number of pneumococci was injected into each individual animal; the dose employed ( $10^{-4}$  cc. of broth culture) was below the zone of true mass infection.

The tests were carried out with the following three groups of mice:

1. The "selected" group of vaccinated animals which had been found in previous tests to have sufficient active immunity to resist infection against at least a small number of Type II pneumococci. These mice had received the vaccination treatment indicated in the preceding description of the procedure employed in obtaining the "selected population." (In addition to the heat-killed vaccine,

they had received an injection of living bacteria in connection with Experiment 1, 2, or 3.)

Five mice from this group were inoculated with the test dose.

2. The "unselected" group of vaccinated animals which, although vaccinated, had never been tested to see whether or not they had developed any immunity. (This group is analogous in this respect to the animals used in Experiments 1, 2, and 3.) The routine of immunization was the same as that described previously under "Methods," with the exception that the mice in this group received one additional course of vaccinations.

Nine mice from this group were inoculated with the test dose.

3. A group of normal animals was included to furnish evidence of the invariability of infection of non-vaccinated mice when invaded by the test dose of

TABLE III.

*Occurrence of Infection in a "Selected" and "Unselected" Group of Vaccinated Mice When Injected with the Same Dose of Type II Pneumococci.*

Groups of mice tested		Number of animals injected	Number survived	Percentage of animals protected
Vaccinated mice	"Selected" group (individuals previously tested for acquired immunity)	5	5	100
	"Unselected" group (individuals not previously tested for acquired immunity)	9	2	22
Normal (not vaccinated) mice	"Selected" group (animals possessing no natural immunity)	6	0	0

bacteria. Six mice received the test dose of  $10^{-4}$  cc.; in addition, the usual duplicate virulence controls were made with  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  cc. of culture.

The results of the experiment are summarized in Table III.

The results of this experiment (Table III) furnish further evidence of the importance of the previous immunity response of the individual in the determination of the occurrence of the postvaccination infections. The two groups of vaccinated mice, although they had received approximately the same previous immunization treatment, represent respectively, an "unselected" and a "selected population." In the "selected" group where the factor of marked differences in individual immunity response had been eliminated, the protection was regular and no infections occurred. On the other

hand, in the "unselected" group (where the actual immunity response of the vaccinated animals had not been tested) some mice were infected and some were not, in spite of the fact that each individual was known to have been invaded by the same, reasonably small number of bacteria. Whether or not infection occurred in the "unselected" group probably depended upon the response of the respective individuals to the previous vaccination.

The chief interest of these results is due to the fact that two factors were controlled which are impossible to control in bacterial infections outside the laboratory: (1) dosage or numbers of the invading bacteria; (2) differences in individual immunity response to the vaccination. When the first of these two factors is constant, the occurrence of the infection depends upon the second factor, and the percentage of infection within the group depends upon the percentage of the individuals that had previously responded effectively to the vaccination. When the second factor is kept constant (as in the "selected population" of known immunes) the occurrence of infection depends upon the numbers of bacteria, and all the individuals are protected if the dosage is kept below the zone of true mass infection.

*Type Specificity of Active Immunity of Mice Vaccinated with Type II Pneumococci.*

The type specificity of the active immunity of mice vaccinated with Type II pneumococci was tested as follows: Four mice were taken from the described "selected population" of Type II-immune individuals and injected respectively with one of the following amounts of virulent Type I organisms,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  cc. of broth culture. (That the animals actually possessed active immunity toward the homologous pneumococci (Type II) was controlled by simultaneous tests of the homologous protection of other individuals from this "selected" group of mice.) Duplicate normal animals were injected with the same amounts to serve as virulence controls of the culture.

The mice which possessed a high degree of active immunity to Type II pneumococci died at approximately the same time as did the normal (unvaccinated) animals, and thus showed no evidence of immunity against the heterologous (Type I) pneumococci. This experiment is of interest as evidence that antipneumococcus (Type II) active immunity of mice is just as type-specific as the immunity in passive protection tests.

## DISCUSSION.

The preceding experiments have dealt with the relative value of dosage and individual immunity response as factors in the infection of mice which had previously been vaccinated with Type II pneumococci. After the completion of vaccination the resistance of the individuals was tested by the intraperitoneal injection of different doses of live bacteria. The results of the tests of active immunity can be analyzed to best advantage according to two zones of dosage.

In the first zone (between  $10^{-6}$  and  $10^{-3}$  cc. of broth culture) the infection or protection of the vaccinated animals was irregular, and certain individuals succumbed to doses much smaller than those which other individuals resisted. The differences in the number of live bacteria injected within this zone seemed to be entirely without influence in the determination of infection or protection since the percentage occurrence of infection was approximately the same in the groups injected with each of the different doses although the maximum dose ( $10^{-3}$  cc.) was 1000 times the minimum dose ( $10^{-6}$  cc.) employed in the experiments. This apparent absence of any relation between size of dose and infection indicates that the irregularities in infection and protection are due to differences in the immunity of the individuals. Since the mice possessed no demonstrable natural immunity to the specific bacteria, the differences in the degree acquired must be referred to differences in individual response to the same vaccination. Hence, in this zone of dosage ( $10^{-6}$  to  $10^{-3}$  cc. of culture), the occurrence of postvaccination infection was determined by the lack of previous immunity response of the individual rather than by the numbers of the invading bacteria.

In the second zone of dosage, the relations were reversed and the injection of overwhelming numbers of the bacteria invariably resulted in infection in spite of the fact that the animals were known to possess a fairly high degree of active immunity. The infection of the mice tested in these controlled experiments can properly be termed true "mass infection;" but it is well to recognize that the same term is often applied to postvaccination infections where the actual immunity of the individual is as unknown as is the dosage.

The above demonstration of the relative value of these two factors

in the infection of mice after vaccination with Type II pneumococci is of some interest in the question of postvaccination infections in general. In our experiments, the factor of differences in individual response to the vaccination was made especially prominent by choosing animals possessing no demonstrable natural immunity and vaccinating them with a type of *Pneumococcus* known not to give a high degree of passive immunity. However, the same factor is probably involved, if much less prominently, in the effectiveness of the vaccination of other animals against other bacteria. The failure of one attack of typhoid fever to render certain individuals immune must be evidence of differences in the immunity response of men to typhoid bacilli. Variations in the passive protective value of convalescent serum from pneumonia and other diseases probably represent examples of the same phenomenon.

#### SUMMARY.

A group of mice was vaccinated against Type II pneumococci and subsequently tested for immunity against different numbers of the live bacteria. The immunity tests were conducted within two zones of dosage. In the first zone where the doses were kept within reasonable limits ( $10^{-6}$  to  $10^{-3}$  cc. of culture), the number of invading bacteria was without influence and the occurrence of infections was determined by the previous immunity response of the individual. In the second zone of dosage (where passive protection also fails), these relations were reversed, and invasion by overwhelming numbers of the bacteria invariably produced infection regardless of the previous immunity response of the individual.

These results present an extreme example of the importance of the immunity response of the individual as a factor always concerned in the effectiveness of vaccination.

# STUDIES ON PATHOGENIC *B. COLI* FROM BOVINE SOURCES.

## I. THE PATHOGENIC ACTION OF CULTURE FILTRATES.

BY THEOBALD SMITH, M.D., AND RALPH B. LITTLE, V.M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

(Received for publication, March 18, 1927.)

The strains used in these studies were all isolated from the ileum of young calves passing liquid feces and showing signs of a choleraform disease (scours). Most of the strains were obtained from sick calves, some moribund, immediately after they had been killed, a few after natural death. Several strains from normal calves were introduced for comparative study. They differ from one another in motility, fermentative capacities, the possession of capsules, tendency to mutate, and the like. More detailed descriptions will be given in papers to follow.

*B. coli*, representing a group, may be regarded as a special type of parasite, restricted to the digestive tube with occasional sallies into those organs or cavities where the protective forces of the host cannot be fully exerted, as in the urinary tract. It was thought that any new light which can be shed on its behavior might be useful in interpreting the behavior of the more highly specialized and parasitic paratyphoid group.

In various articles one of us has formulated the hypothesis that in the gradual evolution of pathogenic or invasive types of bacteria, the beginnings of parasitism may have been made possible by a soluble, diffusible toxin, but that in later stages this primary offensive, more or less accidental, mechanism is either partly or wholly suppressed and some different mechanism developed with which the bacteria protect themselves against the body-foreign forces of the host. The process may be regarded as shifting from the destructive, predatory to the parasitic, from the offensive to the defensive type. According to



this hypothesis, *B. coli* represents the early predatory, toxic stage, with, however, a certain specialization towards protection from anti-foreign activities in the digestive tract. It resembles in many respects the cholera vibrio in its activities. In view of the hypothesis presented, a study of any early appearing toxin in cultures seemed the first problem to be attacked.

#### EXPERIMENTAL.

*Effect of a Living Culture on Calves.*—In the following pages, the effect of culture filtrates on calves is the chief topic. As a preliminary, the notes on the effect of a living bouillon culture are here introduced.

A calf (No. 307), 32 days old, weighing 104 lbs., received into a jugular vein 2 cc. of a 24 hour bouillon culture of *B. coli* 223. This strain produced a large amount of viscid material, even within 24 hours. In 2 days, the entire bouillon became viscid, so that short, cobweb-like threads could be raised from the fluid. The calf was seen 25 minutes later. When seen again, 65 minutes after the injection, it was lying on its side, dead, and a mass of white froth about 12 inches long extended from its nostrils on the floor of the stall. The autopsy showed the following conditions.

Digestive tract pale throughout. Upper respiratory tract, including pharynx and larynx, cyanotic. Tracheal mucosa injected and covered with froth. Lungs large and heavy. Both large caudal lobes are intensely and uniformly congested, the condition bordering on hemorrhage. The condition as to blood content varies from lobule to lobule. The smaller lobes (ventral, cephalic) are far less congested and edematous. Subendocardial tissue, left side, around papillary muscles, infiltrated with blood in form of large patches. Liver with borders rounded. Over large areas there are confluent patches of a dark red color. These, on section, correspond to diffuse hemorrhage into parenchyma similar to the pulmonary lesions. Both kidneys have large dark red areas in the cortex. This hemorrhagic condition dips down in a linear way to medulla. Besides these radiating lines of hemorrhage, hemorrhagic patches are present, as in liver and lungs. Urine from bladder clear, amber-colored, and free from any visible blood tint. It contains a small amount of coagulable protein. In sections of lung, kidney, and liver the described lesions are shown to be due to an intense congestion or filling up of the capillary system associated with and merging into hemorrhage. When 16 days old this calf was fed with a heavy suspension of the same culture without showing disturbance of any kind.

*Effect of Filtrates.*—In a study of filtrates it was deemed best to restrict the experiments on calves to relatively young cultures. Veal broth containing 1 per cent peptone and 0.1 per cent dextrose was

sterilized in flasks in layers 2 to 3 cm. deep. 48 hours after inoculation the cultures were filtered through Berkefeld filters and the filtrate stored in full bottles at 38–40°F. until used. Any deterioration within several months was not observed.

The effect on calves 1 to 2 months old, on calves 6 to 7 months old, and on cows was qualitatively the same when the filtrate was injected into a jugular vein. Subcutaneous injection was without visible effect. After the intravenous injection of 2 cc. of filtrate the first signs in calves were manifest in from 5 to 20 minutes by a slight cough. After this there was a speedy increase in the number of respirations and pulse beats, the former rising to 100, rarely 120 or higher, the latter to 80 or even 100 and above. After 1 or more hours, both declined and the respirations became somewhat jerky. The expirations were usually interrupted and accentuated by a grunt. The temperature rose 1° or 2°C. after the injection but was normal the next day. The respiratory difficulties may in some animals become very great. The mouth is then held open, and saliva dripping from it, the head and neck held horizontal. Usually the calf is very restless, lies down and gets up repeatedly, or when very weak it lies on its side with the legs extended.

The reaction following the intravenous injection is, as might be supposed, not the same in all calves. A few failed to manifest the acute respiratory distress and the reaction showed itself in muscular tremors and chills. In most calves there were repeated discharges of semi-liquid feces in addition to the respiratory symptoms. The pronounced symptoms usually last 4 to 6 hours. Rarely the depression continues over 1 or 2 days. The following protocol is inserted to illustrate the time intervals of the several stages of the toxic effects.

10.20 a.m. Calf, 34 days old, Holstein female, receives, intravenously, 2 cc. filtrate of a 48 hour culture of *B. coli* 1085.

10.25 a.m. Calf very sick, lies down; respirations 130. Coughs frequently.

10.50 a.m. Respirations 88; pulse 60. Respiratory conditions the same.

11 a.m. Respirations 40. Temperature 38.9°C. Pulse 70.

12.15 p.m. Lying with head extended and legs straightened at right angles to body. An expiratory grunt as of some obstruction to expulsion of air.

3.15 p.m. Still lying in the same position. Temperature 39.1°C. A grunt with each expiration but not with any check in the movement. Respirations 70.

4 p.m. Temperature 39.4°C. Still lying down exhausted. Respiration as before. Copious discharge of feces.

5 p.m. Calf standing up. Brighter. Takes its evening food. Temperature 38.9°C.

Calf slightly depressed on the following day. Temperature about normal.

In calves 6 to 7 months old the same dose of 2 cc. produced severe reactions. One cow treated with the filtrates intravenously reacted severely after each injection of a dose increasing gradually to 15 cc. The filtrates of five strains of *B. coli* from calf scours distinguishable culturally from one another all produced the same succession of symptoms.

The mode of introduction of the filtrate naturally brings the respiratory tissues first under the influence of the toxin. The symptoms indicate an injury of the alveolar epithelium and vascular endothelium leading to increased permeability and transudation of fluid into the alveoli. That the toxin is a capillary poison is furthermore indicated by the lesions found in the fatal case following the injection of a living culture described above, and the following cases in which the autopsy showed the end effect on the lung tissue.

Holstein heifer calf, 48 days old, received into a jugular vein, a mixture of 2 cc. *B. coli* filtrate (1192b) and 6 cc. serum from a cow which had been treated with filtrate. This calf had received intravenously 2 cc. filtrate and 2 cc. serum when 38 days old. It went through the typical reaction associated with rapid respiration, open mouth and dribbling of saliva, 35 minutes after the injection. Dyspnea became pronounced and associated with a grunt during each expiration. The labored breathing continued from 11 a.m. until well into the night. The calf appeared free from any respiratory difficulties next morning. It was killed 2 days later. The viscera were normal with exception of the lungs which displayed an irregularly distributed congestion and small hemorrhagic areas. Sections showed the presence of deformed, cup-shaped red cells in small numbers in the alveoli of various regions. Distinct hemorrhagic areas were also present. In some lobules the alveoli contained granular and fibrillated material evidently fibrin. Polymorphs were loosely distributed in small numbers in the alveoli partly enmeshed in the alveolar coagulum. Mitoses of alveolar epithelium were not infrequent. This animal thus was still under the influence of a pulmonary congestion associated with small hemorrhages. There was no evidence of a persisting early or fetal pneumonia.

A Guernsey male calf, 4 weeks old, received into a jugular vein 2 cc. of a filtrate of *B. coli* 223, the same strain which had been fatal to Calf 307, 7 years ago. The filtrate was prepared from a bouillon culture 48 hours old.

The symptoms referable to the respiratory tract followed in the order described but with unusual intensity. The respiratory dyspnea came on within 30 minutes.

The calf breathed with mouth open. The respirations rose to 112, but dropped to 48 after 4 hours. At this time the calf was too weak to stand. The respiratory difficulty increased and the calf died about 5½ hours after the injection. The autopsy immediately after death showed besides a general congestion of the viscera, an intense congestion of the lungs. The right cephalic lobe was completely airless, heavy. On section the parenchyma was evidently filled with blood. The left cephalic lobe contained a little air. The interlobular tissue was broadened by hemorrhagic infiltration. The azygos lobe was in part airless through hemorrhages. Both large caudal lobes showed all degrees of congestion, edema, and hemorrhage from the still air-containing caudal tips to the cephalic margins of these lobes.

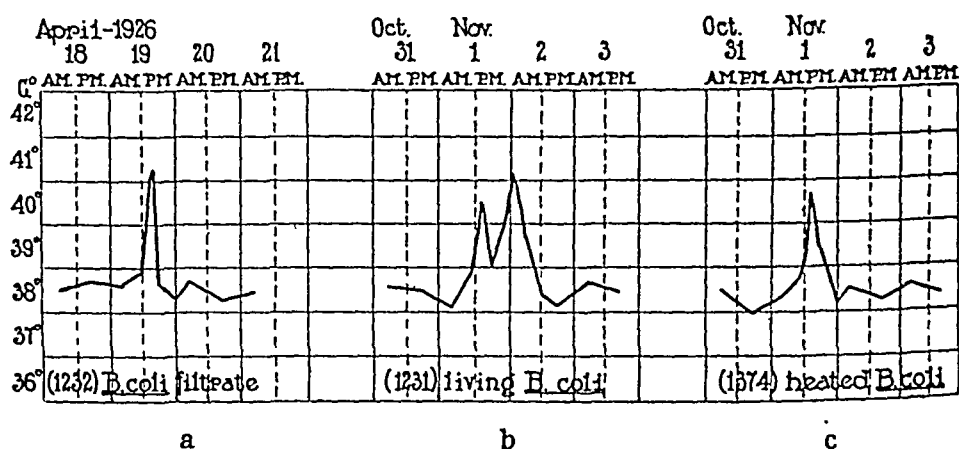
The spleen was enlarged through congestion. The kidneys were moderately congested, with a more deeply injected zone between cortex and medulla. Urine dribbling from the urethra during the early hours after injection of the filtrate was clear, slightly alkaline, specific gravity 1.002, with no protein coagulable by heat. Urine taken from the bladder soon after death had a specific gravity of 1.015, and contained coagulable protein equivalent to a deposit of 1.3 cc. in 10 cc. fluid.

Another calf received subcutaneously, at birth, 40 cc. serum from a cow treated with *B. coli* filtrates. When 29 days old and weighing about 112 pounds, it received into a jugular vein 2 cc. filtrate of a 48 hour bouillon culture of *B. coli* 1192a diluted with 3 cc. bouillon. The filtrate was 49 days old and it had been stored continuously at 36–40°F. in full bottles. Symptoms began within 20 minutes and ran the usual course with panting respirations rising to 120 per minute. The calf stood with head low, mouth open, and tongue protruding. 1½ hours after injection the respirations had fallen to 72. The animal was then very sick and unable to stand. Grunts with every expiration. 3½ hours after injection the animal began to be easier. Respirations 60, with occasional grunts. In 6 hours the reaction was nearly over and 1 hour later the evening meal of milk was taken readily.

The calf was killed about 23 hours after injection when in outwardly normal condition. The vessels of neck were severed after stunning the animal. The only organs visibly changed were the lungs. These were extensively involved. Three forms of lesions could be observed. (a) A dark red hepatization due to exudation into alveoli of blood and coagulation there. About 8 cm. of the free tip of the left cephalic lobe was in this condition. (b) Localized congestions and hemorrhages involving one or several lobules. These were scattered through the main (caudal) lobes chiefly. (c) Petechial hemorrhages 1 to 3 cm. apart, chiefly in the small cephalic lobes. The liver showed a patchy congestion visible both on surface and on section. Each liver cell contained one or more fat globules 2 to 5μ in diameter. Kidneys slightly congested and distinctly more moist on section than normal. Hyperemia of boundary zone between cortex and medulla. Spleen congested but normal markings still visible. Slight patchy hyperemia of upper small intestine. Urine about 30 minutes after injection contained about

1.3 per cent deposit of coagulable protein after heating. At autopsy there was only about 0.3 per cent. Urine 12 days before injection was normal.

Sections of fixed and hardened tissue presented nothing noteworthy beyond what is stated in the autopsy notes concerning the liver, spleen, and kidneys. In sections of the lungs, that portion found consolidated at autopsy presented various lesions. Focal hemorrhages were numerous and filled a group of contiguous alveoli with red cells. Forming a background for these hemorrhagic areas, the remainder of the alveoli contained a delicate network of fibrin fibrils holding enmeshed polymorphs in small numbers. The latter cells were brought together in denser groups within alveoli here and there. The other lobes of the lungs showed areas with partly collapsed alveoli and broadened walls as well as occasional small hemorrhages and alveolar fibrin. Polymorphs were thinly distributed throughout, both in the capillary bed and the alveolar lumina.



TEXT-FIG. 1. Temperature reactions in calves following the intravenous injection of *B. coli* and filtrates.

Thus far only filtrates of 48 hour bouillon cultures had been used. The presence of abundant toxin in a 24 hour culture was demonstrated on a calf 26 days old. After the intravenous injection of 2 cc. of the filtrate the sequence of symptoms already described appeared and with an intensity fully equal to that following the 48 hour culture filtrate. The calf was killed within 48 hours of the injection. The lungs still presented signs of the reaction in the form of subpleural hemorrhagic points and scattering congested lobules.

The effect of the intravenous injections on the rectal temperature of calves is shown in Text-fig. 1. The effect of a filtrate of a 48 hour bouillon culture is shown in (a). A similar unimodal curve (c) is pro-

duced by cultures heated at 60°C. for 30 minutes and therefore sterilized. In (b) the bimodal curve following the injection of living cultures may be due to immediate effects of the toxin followed by a temporary multiplication of the injected bacteria and hence a second dose of toxins.

The experiments reported were made with 24 and 48 hour culture filtrates. One test was made with a filtrate of a culture of 1192a incubated 8 days. The layer of bouillon in the flask was about 1 cm. deep. At the end of the incubation, the fluid was quite viscid and hence diluted with an equal volume of normal saline to facilitate passage through a Berkefeld filter.

4 cc. of the filtrate, containing 2 cc. of the original culture fluid, was injected into a jugular vein of a calf 34 days old. The symptoms followed one another as in the preceding cases but with much greater intensity and rapidity. The calf died in 3½ hours after the injection. There was complete hemorrhagic filling up of the entire left lung excepting a narrow margin of ventral and cephalic lobe. On section, the tissue was uniformly dark red, with reddish frothy fluid trickling off. The tissue was heavy but still resilient. The right lung, along median and dorsal region, was in the same condition; the lateral two-thirds of this lung was still partly air-containing with dark red areas in each lobule. Much foamy reddish fluid flowed from cut section of the pinkish regions. The trachea was filled with a reddish froth. There was a moderate congestion of the kidneys and some fat in the liver cells. The mucosa of intestines was only feebly reddened. The spleen was congested and weighed 460 gm.

This preliminary test clearly indicated a rise in the toxicity of the culture fluid due to longer incubation. Whether there is but one toxin involved or others superadded during the longer incubation remains unanswered for the present.

*The effect of filtrates on guinea pigs* introduced into the peritoneal cavity is relatively slight when compared with the serious effect on calves weighing about 100 pounds. The guinea pig weighing 350 to 600 gm. receiving the calf dose of 2 cc. into the peritoneal cavity reacts only with loss in weight as follows: Within 2 days there is a loss of 35 to 50 gm. in weight. Then there is a recovery so that in 7 days the original weight has not only been regained but added to by 10 to 15 gm. A small portion of this loss may be produced with bouillon alone. The injection of smaller doses, up to 0.5 cc., directly into the

heart failed to produce any acute symptoms or later death. In relation to body weight 0.5 cc. into the circulation of guinea pigs is over 30 times, the intraperitoneal dose of 2 cc. over 120 times the calf dose. In an early, fairly comprehensive study of calf scours, E. Joest<sup>1</sup> finding that filtrates of 24 hour bouillon cultures of *B. coli* were non-toxic for guinea pigs after intraperitoneal injection up to 3 cc., concludes that soluble poisonous products are not secreted.

It has already been stated that the subcutaneous injection of filtrates is without appreciable effect. The same is true when living cultures are introduced with the food. Two calves, about 2 months old, were fed *B. coli* in milk without showing any digestive or other disturbances. 500 cc. milk had been heavily seeded with a bouillon culture, warmed, and incubated for 7 hours. Plate cultures indicated  $\frac{1}{2}$  billion bacteria per cc. To disguise the flavor, fresh milk was added. The incubated milk coagulated when heated and was strongly acid to litmus.

Owing to obvious difficulties in multiplying experiments on calves only a few have been made bearing other phases. When bouillon cultures were shaken with kaolin and filtered there was no reduction of toxicity. When the filtrate was exposed to 80°C. for 30 minutes the toxicity though decidedly reduced was not completely destroyed.

One calf was treated with a 48 hour culture filtrate of a paratyphoid bacillus from guinea pigs.<sup>2</sup> 4 cc. in place of the usual 2 cc. were injected into a jugular vein. The symptoms following were similar to those produced by the *B. coli* filtrate, but less severe. The calf began to cough in 13 minutes. In 15 minutes the respirations were 104. In 30 minutes the calf was lying down, with respirations at 80 and evidently labored. After 1½ hours, respirations were 60 and dyspnea pronounced. Viscid saliva was hanging from the mouth. After 3 hours, the calf was lying quiet and apparently without distress. It took its milk after 6 hours. Next day it was still subdued. During the attack the temperature rose 1-1.5°C.

The soluble toxins of the large group of typhoid, paratyphoid, and colon bacilli have interested many observers since these groups were definitely recognized. Much of the work has been done in the paratyphoid group owing to its close relation to outbreaks of meat and

<sup>1</sup> Joest, E., *Z. Tiermed.*, 1903, vii, 377.

<sup>2</sup> Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353; and Smith, T., and Nelson, J. B., *J. Exp. Med.*, 1927, xlv, 365.

other food poisoning. A fairly complete bibliography has been published recently by Miss Branham.<sup>3</sup> Among the recent papers which bear directly on the subject of this communication is one by Steinberg and Ecker<sup>4</sup> who prepared an antiserum in rabbits towards the soluble toxin with a culture fluid centrifuged but not filtered. The antiserum was tested upon rabbits inoculated with living cultures. The results apparently demonstrated the neutralizing power of the antiserum on the soluble toxin. An analysis of the experiments does not bear out the inference drawn. The rabbits received some living bacteria as antigen and the effect of the antiserum in the test rabbits may have been a suppression of the bacteria injected rather than a neutralization of the soluble toxin.

#### SUMMARY AND CONCLUSIONS.

The relatively young bouillon filtrates, 24 and 48 hours old, of certain strains of *B. coli* obtained directly from the ileum of scouring calves, were highly toxic for calves about 1 month old, as well as for older calves and cows when given into a vein. The symptoms, of panting followed by dyspneic and jerky respiration, indicate some at first obstructive action upon the alveolar and endothelial cells, followed by a greater permeability and eventual filling up of the air spaces with a serous, fibrinous, and hemorrhagic exudate. Similar effects are produced in other organs, such as liver and kidneys, if the toxin reaches them or is formed there by multiplying bacteria. There are no immediate or remote effects resembling those on calves following the intraperitoneal or the intracardiac injection of *B. coli* filtrates into guinea pigs even when the dose represents many multiples, per body weight, of the dangerous or even fatal calf dose.

The administration of the filtrate subcutaneously is without visible effect. Similarly, feeding large numbers of living bacilli produced no manifest disturbances.

In support of the hypothesis of a genetic relation between the group of *B. coli* and of paratyphoid, a similar but less severe effect was produced in a calf by the intravenous injection of a bouillon filtrate of a paratyphoid strain.

<sup>3</sup> Branham, S. E., *J. Infect. Dis.*, 1925, xxxvii, 291.

<sup>4</sup> Steinberg, B., and Ecker, E. E., *J. Exp. Med.*, 1926, xliii, 443.







expansions was not confined to one race. The strains figured differ from one another in one or more characters. Thus No. 223 is a motile, saccharose-fermenting strain; Nos. 1192, 1127, and 1085 are non-motile, non-saccharose-fermenting strains. All differ from one another in degrees of virulence.

The mutants obtainable on agar plates from lateral expansions of the colonies will be called (b) in the following pages, and the original nucleus of the colony (a). This mode of designation leaves open the use of other letters for mutants obtained in other ways. Only the (a) and (b) types will be considered in this paper.

Although morphological differences between (a) and (b) forms have not been observed, a definite distinction exists due to the presence or absence of an optically distinct capsule associated with a viscid condition of the growth in solid and in fluid media. The capsule was found only on (a) forms. Focusing on the periphery of the hanging drop of a diluted agar or a bouillon culture, one can frequently see the capsule, or a distinct spacing of the rods as they crowd together in the border of the drop. This spacing disappears as the marginal mass of bacteria begins to dry. The viscosity may be roughly estimated by the length of the thread which can be lifted up from agar or bouillon cultures after 1 or 2 days. The loss of viscosity and capsule occurred in all (b) forms studied. The presence of capsular substance was signalized by a heavy, glistening growth layer on sloped agar. This in some strains led to a slow sliding down of the growth from colonies on the slope, producing broad, vertical streaks. Such appearances may be seen in cultures of *B. lactis aerogenes* and some strains of the Friedländer bacillus. In (b) types, the growth was thinner, partly translucent, and easily distinguished on sloped agar from the (a) types. It did not form descending streaks of growth from colonies.

In bouillon after 1 or 2 days, (a) forms produced degrees of viscosity, evident, if not by the formation of cobweb-like threads at the end of platinum loop or wire, at least by the tardiness with which air bubbles rose to the surface when the fluid was shaken. These features were absent in (b) cultures. Fermentation reactions were not changed by the mutation, nor was there loss or gain of motility.

*Immunological Characters.*—Marked differences in the relation of (a) and (b) forms towards animal hosts were in evidence in all strains

studied. (a) forms are agglutinated only in very low dilution in the sera of animals treated with them or else not at all. This difference in agglutinability was first observed in rabbits immunized with killed cultures. Later rabbits were treated with both (a) and (b) types. In one experiment (a) serum agglutinated the (a) form in a dilution of 1:40, the (b) form in a dilution of 1:40,960. A rabbit treated with the (b) form produced a serum which agglutinated the homologous form at 1:40,960, but did not influence the (a) form at all.

To obtain sera from larger animals, the cow, being the normal host of the types of *B. coli* under investigation, was chosen. The first cow (1109) was treated with living 24 hour bouillon cultures of *B. coli* 1127a over a period of about 5 months. The injection was chiefly intravenous, the initial dose about 1 cc. The reaction was always fairly severe (1) so that the increase in dosage was very gradual. The final dose was 17 cc. Agglutination of the (a) form was barely indicated at 1:20, whereas the limit of agglutination of the (b) form was 1:1,280. A second cow was treated in a similar way with living cultures of 1192a. After 6 months of treatment with weekly injections the dose safely endured was 10 cc. Agglutination tests with this serum were negative towards the homologous culture at a dilution of 1:10. Towards the (b) form, however, clumping was nearly complete at 1:640, the highest dilution tried. The (b) form was clumped in high dilution by the immune cow serum of Strain 1127a and this serum agglutinated the (b) form of 1192. Serum of a normal horse caused complete clumping at 1:640, the highest dilution tried. Serum of a normal cow clumped completely at 1:80 (b) strains of both 1192 and 1127. Serum of a normal rabbit, however, failed to clump at 1:10. Serum of a normal calf (1242) showed slight clumping of (b) in a 1:40 dilution. The mutation process had so changed the (a) form that it became agglutinable in high dilutions not only in homologous immune serum but also in sera of untreated cow and horse. Cross-agglutination with (b) forms of *B. coli* strains showing wide differences in virulence towards guinea pigs was observed. The agglutinins in horse and cow blood may have been due to the accumulation of antibodies resulting from intestinal multiplication of various types of *B. coli*.

To determine the relation of leucocytes to the (a) and (b) forms the

blood of young calves was tested. The Wright technique was used and the capillary pipette containing the mixture of blood and bacteria incubated for 10 minutes at 37°C. The phagocytic index was depressed by the use of citrate, and was highest when defibrinated blood was substituted. Although the results were irregular there was no exception to the findings that the calf polymorphs were nearly inactive in the presence of the (a) type, but the (b) type was readily taken up. The presence of agglutinins in high dilution in the immune rabbit serum towards (b) organisms interfered with its use in the phagocytic test.

*Virulence of (a) and (b) Forms.*—Early in 1925 a study of the virulence of living cultures of various strains of *B. coli* from scours towards

TABLE I.  
*Virulence of the Original and the Mutant Form Compared.*

Strain of <i>B. coli</i>	Minimum fatal dose		$\frac{(b)}{(a)}$
	cc. (a)	cc. (b)	
1085	0.04	1.0	25.0
1127	0.2	0.7	3.5
1192	0.04	1.0	25.0
223	0.06	0.7	11.6

guinea pigs was begun. The intraperitoneal route was chosen since subcutaneous injections failed to furnish any measurable data. The injection of the minimum fatal dose causes death within 24 hours. Very rarely an animal dies during the 2nd day. The reaction thus simulates the effect of a soluble or exotoxin. A comparative study of the minimum fatal dose of 24 hour bouillon cultures of (a) and (b) types yielded results shown in Table I. The last column expresses the relation between the minimum fatal doses of (a) and (b) forms. It will be noted that in these strains the minimum fatal doses of the (b) forms differ but little from one another, whereas the (a) forms show a greater spread.

Virulence in (a) types remained relatively constant when growths on sloped agar were transferred once in 4 to 6 weeks, incubated over-

night, and placed at 38–40°F. One strain (223) kept for 8 years in the manner described was still highly virulent for guinea pigs. In harmony with this persistence of virulence mutants did not appear in stock cultures kept in the manner described even after years of cultivation as repeated platings have shown.

The toxin production of (a) and (b) types was tested on calves as described (1). Two strains were used, Nos. 1069 and 1192. Given the same dose intravenously the characteristic respiratory symptoms were produced with equal severity by (a) and (b) filtrates of these two strains.

The uniform tendency of *B. coli* during mutation to lose certain characters indicating virulence and to acquire others also indicating loss of virulence marks this process as one of degeneracy but with reference to parasitism only. The degraded form persisted as such in cultures indefinitely. Two attempts were made to either restore or at least to modify it. One strain (1192b) was passed through a series of nine guinea pigs. Some of these died, others were chloroformed in 48 hours. Cultures were made from the peritoneal cavity and injected into the next of the series when 24 hours old. No increase in virulence or reappearance of viscid or capsular state was observed at the end of the series. A second attempt was made to change the mutant by passing it twice a day through bouillon tubes until more than 50 transfers had been made. At the end no changes towards the (a) form were manifest (2). The possibility of bringing the mutant back is not exhausted by these procedures. Not until such strains have been passed through the digestive tract of calves where the increased virulence of the (a) form may have been developed originally may we regard the change as irreversible.

#### DISCUSSION.

An examination of the voluminous literature on *B. coli* brings out certain data anticipating those briefly described. Baerthlein (3) in his extensive study of mutations of various species of bacteria mentions one group of paratyphoid strains, including two *suipestifer* and one *psittacosis* strain, which mutated on agar by sending out after several days a thinner, more translucent zone. In most writings the actual development of mutants from the original colony was not seen.

We are left to infer that cultures kept under certain conditions for a given period when plated presented two and sometimes more colony types. It is highly probable that the mutational changes went on in cultures kept in room temperature, exposed to diffuse light, and slowly drying out, although this is not stated. Massini (4) who was the first to observe mutations obtained his mutants from colony out-growths resembling knobs (*Knöpfe*). No reference was found to the loss of capsules, or the disappearance of a viscid product in mutant forms.

Gratia (5) isolated ten mutants from a single original stock of *B. coli*. These differed from one another in motility and mucoid growth. The mucoid condition in his strain was a mutational state and not a property of the original strain as in our *B. coli* types. Changes in morphology for the cholera vibrio, typhoid, paratyphoid, dysentery, and colon bacilli are described by Baerthlein (3). One form appeared plump, the other slender.

Modification of agglutinability due to mutation was observed by Gratia in his colon strain and by Baerthlein in *B. enteriditis*. His (b) forms were agglutinated more easily than (a) forms. The (b) agglutinins were absorbed with great difficulty, if at all. In a *B. coli* mutant, the agglutination presented much the same peculiar conditions as we have described them for (a) and (b) forms. According to Baerthlein no changes in agglutinability were noted in the cholera vibrio and in typhoid and dysentery bacilli. De Kruif (6) noted changes in acid agglutination but not when immune serum was used (7) in his studies on dissociation in the rabbit septicemia organism. Reimann (8), in studies of pneumococci, finds the S or original form as type-specific, the R or mutant form as group-specific. The former is agglutinable only in its type serum; the latter in its own and in S serum. The distinction of type and group agglutinin (Reimann) was possibly foreshadowed by Burk (9) who found *B. coli* races from the human intestine agglutinated by sera from a variety of normal animals including rabbits. Mutation as interpreted by Baerthlein is more or less temporary and forms may pass back from mutant to original type. He does not touch upon virulence. Later studies by others indicate that mutation, in pathogenic forms at least, signifies a degradation with reference to virulence or parasitism. This view is taken by De Kruif

and Reimann and is clearly indicated in our results with *B. coli*. Recovery of the original level of virulence did not occur in cultures and not in passages through animals. As stated above a reversion to the (a) type in the original host animals is not negatived by experiments made thus far. Concerning changes in the character or concentration of toxins no statements have been found.

This brief and incomplete review suggests that the term mutation has been used to designate a variety of changes which bacteria may undergo during artificial cultivation. Some are obviously degradations and involve permanent loss of certain functions. Some are probably exaggerations or depressions of functions which can be brought back by certain procedures, as for instance the change back from R to S type by rapid transfers in bouillon (2).

The task of the future will be to redefine and classify the changes or mutations and evaluate them from different points of view. Especially desirable is a renewed study of those mutations in which definite functions are acquired, such as motility, fermentation of lactose (4), and of saccharose (10). These changes may be regarded as depressions or exaggerations or inhibitions rather than as new acquisitions until more exacting methods have been applied in a study of them.

#### GENERAL CONCLUSIONS.

On agar plates certain strains of *B. coli* from the ileum of calves suffering from diarrhea or scours promptly mutate and give rise to forms which have lost capsular substance, whose virulence has been greatly reduced, and which have gained very greatly in agglutinability and in being taken up by leucocytes. The original characters are not regained in cultures kept in the cold after development, nor in rapid transfers in bouillon, nor in passages through the peritoneal cavity of guinea pigs. Filtrates of 48 hour bouillon cultures contain as much toxin in the (b) as in the (a) form indicating no loss in this function.

#### BIBLIOGRAPHY.

1. Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, xlv, 123.
2. Jordan, E. O., *J. Am. Med. Assn.*, 1926, lxxvi, 176.
3. Baerthlein, *Arb. k. Gsndhtsamte*, 1912, xl, 433.



4. Massini, R., *Arch. Hyg.*, 1907, lxi, 250.
5. Gratia, A., *J. Exp. Med.*, 1922, xxxv, 287.
6. De Kruif, P. H., *J. Gen. Physiol.*, 1922, iv, 387.
7. De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.
8. Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 107.
9. Burk, A., *Centr. Bakt., 1. Abt., Orig.*, 1908, xlv, 577.
10. Burri, R., and Dügge, M., *Centr. Bakt., 1. Abt., Orig.*, 1909, xlix, 145.

#### EXPLANATION OF PLATES.

##### PLATE 6.

FIG. 1. Agar plate of *B. coli* 1127a. After several days incubation the outgrowths were nearly of maximum size and continued to expand but slightly in room temperature under a darkened bell glass. The plate was photographed after 21 days.

FIG. 2. Agar plate of *B. coli* 1085a. Details as for Fig. 1.

##### PLATE 7.

FIG. 3. Agar plate of *B. coli* 1192a. Details as for Fig. 1.

FIG. 4. Agar plate of *B. coli* 223. On this plate a different form of outgrowth is illustrated. The mutant appears as a web-like expansion between clefts formed in the original colony.

All four plate cultures were prepared and photographed at the same time.

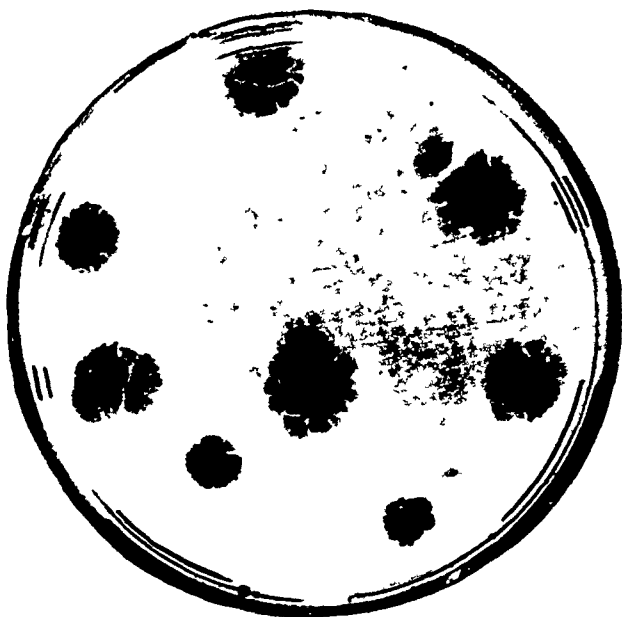


FIG. 1.

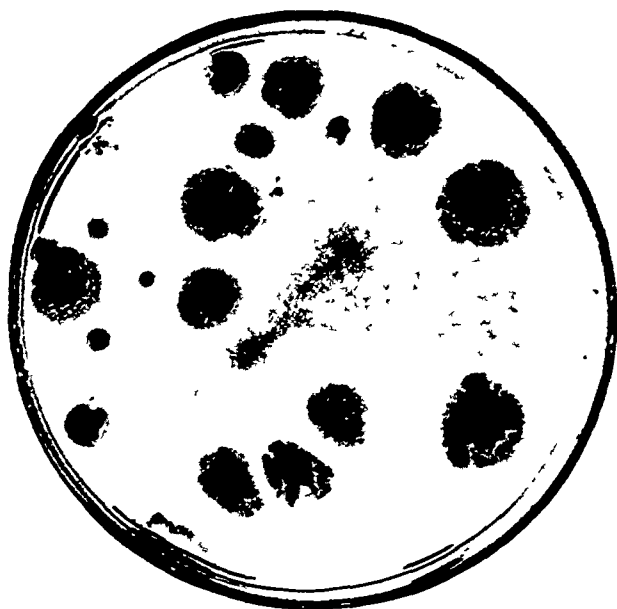


FIG. 2.

(Smith and Bryant. Pathogenic *B. coli* from bovine sources. II.)



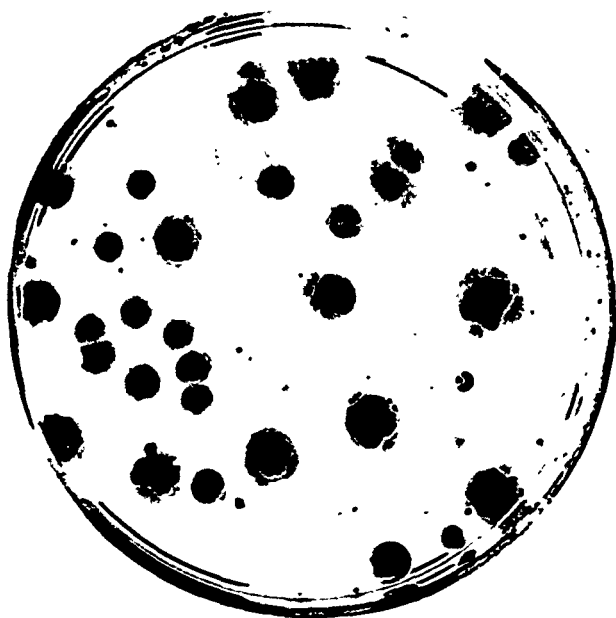


FIG. 3.

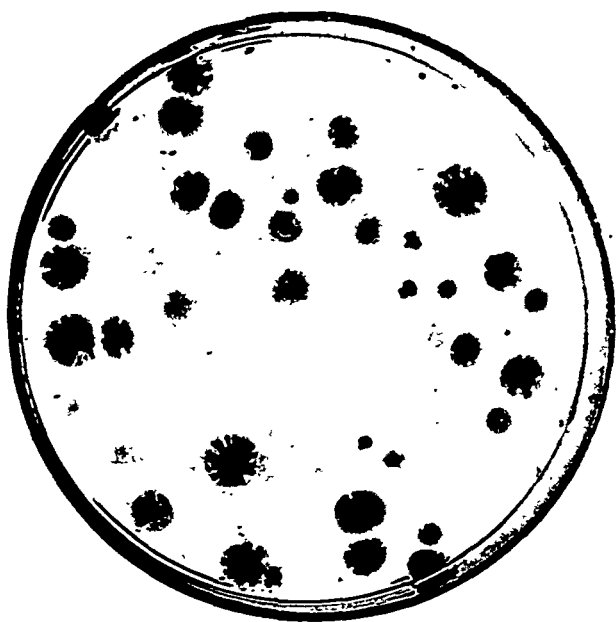


FIG. 4.

(Smith and Bryant Pathogenic *B. coli* from bovine sources. II.)



## STUDIES ON PATHOGENIC *B. COLI* FROM BOVINE SOURCES.

### III. NORMAL AND SEROLOGICALLY INDUCED RESISTANCE TO *B. COLI* AND ITS MUTANT.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, March 18, 1927.)

In view of the formidable differences in serological behavior of the original and the dissociated or mutant forms of *B. coli* described as appearing within 48 hours on agar plates made directly from intestinal contents,<sup>1,2</sup> it became desirable to analyze the virulence of the original and the mutant strain with the use of immune sera. To do this it became necessary to use the guinea pig in place of the cow or calf. This animal has been used in the past by various workers beginning with the early studies of the cholera vibrio by R. Pfeiffer. The intraperitoneal injection was chosen since the subcutaneous route fails to elicit any measurable reaction. To determine the minimum fatal dose bouillon cultures just 24 hours old were used. In these the turbidity of different strains and of the same strain in different lots of bouillon was markedly uniform and any more elaborate measure of dosage of the bacteria was dispensed with. The plating of definite dilutions of 24 hour bouillon cultures of the strain used chiefly in the experiments to be reported showed that there were present in 1 cc. 900 million of *B. coli* 1192a and 1 billion of the mutant (b).

The disease in guinea pigs following the intraperitoneal injection of the highly virulent or (a) form<sup>2</sup> was characterized by a prompt offensive and defensive reaction leading to death within 24 hours, or else only quiescence for 12 to 18 hours, loss in weight, and recovery

<sup>1</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, xlv, 123.

<sup>2</sup> Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlv, 133.

within 2 or 3 days. Very rarely an animal died after 18 to 24 hours. The minimum lethal dose may be titrated with nearly the same accuracy as is possible with diphtheria toxin. The effect of non-lethal doses may be roughly gauged by loss in weight. In early deaths, occurring between 6 and 10 hours, there is a trace of bloody fluid in the peritoneal cavity and indications of hemolysis. Certain viscera lying against the dorsal wall, such as the uterine horns and dorsal wall of cecum, may be spotted with hemorrhagic areas. Bacteria coat the peritoneum in large numbers. In animals dying between 12 and 24 hours, there is less congestion and hemorrhage and instead a beginning filmy exudate on liver and spleen. Bacteria may be very numerous or absent in films. All gradations are found between these extremes.

With the appearance of exudates, the viscera become covered with a translucent film of cells, not noticed at first until cover-slips are laid on the peritoneum and drawn away. Polymorphs are the only cell form at first and they increase in numbers with prolongation of life. When death occurs, in about 24 hours, there may be  $\frac{1}{2}$  to 1 cc. of a ropy, opalescent fluid made up of leucocytes and bacteria.

In surviving guinea pigs, macrophages appear near the end of the first 24 hours and gradually increase in numbers so as to represent from one-fifth to one-half the number of cells present. Bacteria are very scarce at this stage. The cell exudate may be traced for 3 or 4 days, after which period it disappears. Occasionally whitish, viscid masses, 2 to 3 mm. in diameter, made up of polymorphs and macrophages remain in the folds of the omentum and spaces between the viscera. The lungs, at first normal in appearance, become greatly congested if death occurs as late as 18 to 24 hours. Usually from  $\frac{1}{2}$  to 2 cc. of clear fluid is present in the pleural cavity at this time. Recovered guinea pigs, killed in 2 or more weeks, frequently have the spleen bound down by one or more firm adhesions to the abdominal wall.

The normal guinea pig weighing 350 to 400 gm. is capable of destroying a certain number of *B. coli* introduced into the peritoneal cavity. Among the strains isolated from calves the minimum fatal dose varied from 0.02 cc. to 0.5 cc. of a 24 hour bouillon culture. As a rule, three-fourths of this dose was still overcome by the guinea

pig. There was, however, a considerable fall in weight—from 40 to 50 gm.—when doses near the limit were injected, with a recovery of the original weight in 7 to 10 days. The injection of living *B. coli* thus gives almost as sharp a death point as is obtainable with diphtheria toxin. Of those that die, death occurs within 24 hours in fully 95 per cent. The bulk of the bacteria injected disappear from films within 24 hours, even in the rare cases that die after this time. In cultures from such animals made with loops rubbed over the viscera or dipped into traces of fluid present, usually a few colonies appear. In guinea pigs killed 2 or 3 days after inoculation, bacteria may frequently be cultured from the small clumps of cellular exudate filling interstices between viscera. After 4 or 5 days, these also are sterile.

Phagocytosis by polymorphs is evident between 12 to 24 hours, even in fatal cases, and increases with time. It is more pronounced following the injection of less virulent races. By the time macrophages appear, bacteria are scarce or absent. These cells are seen containing bacteria in very rare instances. In view of the usual function of macrophages in this experimental disease—ingesting polymorphs—the contained bacteria are probably within polymorphs in the macrophages. This is indicated when neutral red is used as a vital stain since the latter brings out the polymorph inclusions.

When a fraction of the fatal dose of a (b) mutant<sup>2</sup> of low virulence is given, the process followed by killing the animals at intervals indicates a rapid destruction of bacteria. When one-fourth the fatal dose is injected, representing about 0.25 cc. of culture fluid and equivalent to perhaps six times the number of bacteria in the fatal dose of the original or (a) strain, the number of living bacteria in the abdominal exudate falls to one-third within an hour. A small number may survive for 2 or 3 days in the lumps of polymorphs attached to omentum or viscera. The movement of polymorphs into the peritoneal cavity is more rapid in the presence of mutants and within 4 hours the peritoneal fluid is clouded with clumps of such cells. The cellular reaction beyond being more prompt does not differ in its further behavior from that already described for the (a) strain.

The toxicity, which maintains itself quite uniform in the same lot



of bouillon, must be retested when new lots of bouillon are used. If meat (veal) from young animals of nearly the same age is used the change from lot to lot is slight. If, however, meat from yearlings or older cows is used, the result may be quite different. In the case of Strain 1192a, the minimum fatal dose fell from 0.05 cc. to 0.02 cc. when meat from an older animal was used. Table I gives the results of one of several tests between the latter and a fresh lot of veal bouillon.

*The Action of Immune Sera in the Protection of Guinea Pigs.*—To study the factors involved in the varying behavior of *B. coli* in the peritoneal cavity of the guinea pig, immune sera were introduced into the problem. To obtain a fairly active serum cows were immunized. In what follows the sera of three cows were used. A

TABLE I.  
*Toxicity of Cultures Made from Different Lots of Meat.*

No. of guinea pig	Weight	Dose ( <i>B. coli</i> )	No. of bouillon	Results
	gm.	cc.		
1	375	0.02	2252b (veal)	Lives
2	380	0.024	2252b "	"
3	375	0.02	2239 (beef)	Dead in 11 hrs.
4	380	0.024	2239 "	" " 11 "

brief statement of two of these will be found in an earlier publication.<sup>2</sup> No. 1109 was treated intravenously with living 24 hour bouillon cultures of *B. coli* 1127a. No. 1231 received intravenous doses of *B. coli* 1192a, and No. 1232, only Berkefeld filtrates of 48 hour bouillon cultures of 1192a. A more complete history of these cows will be given in another publication.

The sera thus produced were tested on guinea pigs.<sup>3</sup> The minimum fatal dose of a 24 hour bouillon culture of 1127a injected into the peritoneal cavity of a guinea pig weighing 350 to 400 gm. was 0.2 cc. Of the homologous immune serum, 0.1 cc. mixed with the culture was the minimum life-saving dose.

<sup>3</sup> The method here employed of injecting a mixture of culture and serum into the peritoneal cavity of the guinea pig had been used by Grosso in testing scours sera. See Grosso, G., *Z. Infektionskrankh. Haustiere*, 1912, xii, 54.

The marked protective action of colostrum and of cow serum administered to new-born calves suggested the idea that normal cow serum might in itself neutralize the fatal effect of *B. coli* in the guinea pig. In Table II is shown a comparative test with specific immune serum (*B. coli* 1127a) and normal cow serum. The immune serum protected against the minimum fatal dose in 0.1 cc. but not in 0.05 cc. amounts. The normal serum failed to protect in 1 cc. doses and a later test with 1.5 and 2 cc. did not retard death. Similar tests with sera from four other cows in doses of 1 to 2 cc. failed to

TABLE II.

*Effects of Normal Serum (Cow 1100) and Immune Serum (Cow 1109).*

Weight of guinea pig	24 hr. bouillon culture	Serum	Source of serum	Remarks
gm.	cc.	cc.		
450	0.2	—	—	Dead in 11 hrs.
460	0.2	0.1	Cow 1100	" " 12 "
465	0.2	0.2	" 1100	" " 11 "
440	0.2	0.1	" 1109	Lives
430	0.2	0.2	" 1109	"
525	0.2	0.05	" 1109	Dead in 12 hrs.
550	0.2	0.1	" 1109	Lives
510	0.2	0.2	" 1109	"
410	0.2	0.5	" 1100	Dead in 12 hrs.
425	0.2	0.7	" 1100	" " 8 "
440	0.2	1.0	" 1100	" " 12 "
490	0.2	—	—	" " 12 "

retard death. In these different tests, the sera had been stored for some time and any toxic effect of the 2 cc. doses was not noticed.

Strain 1192a was fatal to guinea pigs of the same weight in doses of 0.05 cc. In a preliminary test the serum of the cow treated with living bacteria saved life in 0.5 cc. doses. The "filtrate" serum failed to do this in the same dose. Later tests demonstrated that 0.05 cc. of the living-culture serum protected against 0.06 cc. culture, a trifle more than the minimum fatal dose. Table III illustrates the action of the immune serum at this stage.

A continuation of the treatment of both cows resulted in stronger immune sera. That of the animal treated with intravenous doses of

living cultures 11 months protected guinea pigs in doses of 0.005 cc. against  $1\frac{1}{4}$  times the surely fatal dose. The serum of the cow treated with filtrates failed to do this in doses less than 0.5 cc. The law of multiples effective with diphtheria toxin and antitoxin mixtures fails when applied to the pathogenic action of living cultures of *B. coli*. In several trials, after the minimum serum dose effective in neutralizing  $1\frac{1}{4}$  times the minimum fatal dose of culture fluid had been established as 0.005 cc. serum protecting against 0.025 cc. culture, a dose of 0.2 cc. serum, or 40 times the above dose, protected against

TABLE III.

*Effects of Immune Cow Sera 1231 (Living Culture) and 1232 (48 Hour Culture Filtrate), Strain 1192a.*

Weight of guinea pig	Dose of culture	Dose of serum	Result
<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	
350	0.04	—	Lives
355	0.05	—	Dead in 15 hrs.
370	0.06	—	" " 10 "
350	0.05	0.5, No. 1231	Lives
355	0.06	0.5, " 1231	"
370	0.075	0.5, " 1231	"
340	0.06	—	Dead in 5 hrs.
340	0.06	0.2, No. 1231	Lives
340	0.06	0.3, " 1231	"
350	0.06	0.1, " 1231	"
375	0.06	0.05, " 1231	"
350	0.06	0.5, " 1232	Dead in 8 hrs.
340	0.06	1.0, " 1232	" " 8 "

0.1 cc. culture, or 4 times the fatal dose. 0.1 cc. serum did not protect. Assuming in the latter case the protective dose to be actually 0.15 cc. serum, 4 times the minimum fatal dose requires 30 times the minimum serum dose.<sup>4</sup>

Although the immune cow sera showed a very definite protective action on guinea pigs, they failed to neutralize the toxic action of

<sup>4</sup> If we regard 0.02 cc. culture as controlled by the natural resistance of the guinea pig and subtract this from the culture doses used, we still have a culture ratio of  $\frac{0.08}{0.005}$ , or 16, to a serum ratio of  $\frac{0.15}{0.005}$ , or 30.

filtrates on calves. The sera were tested on fifteen calves. The dose of filtrate was 2 cc., that of the sera from 2 to 6 cc.

The respiratory symptoms already described<sup>1</sup> as produced by 2 day filtrates of both (a) and (b) forms of *B. coli*, were not neutralized or modified to any extent when mixed with either "living culture" or "filtrate" serum before injection. Thus the dose of filtrate usually injected, when mixed with 2 cc. and 3 cc. homologous "living" serum (1127a) failed to limit appreciably the violent reaction. The

TABLE IV.

*Effect of Cow Serum (B. coli 1192a) on Pathogenic Action of Four Other Strains.*

Weight of guinea pig	Strain	Culture dose	Serum dose	Result
gm.		cc.	cc.	
365	223a	0.07	—	Dead in 14-16 hrs.
360	223a	0.06	—	" " 24 "
365	223a	0.07	0.5	Lives
360	1085a	0.04	—	"
385	1085a	0.05	—	Dead in 14-16 hrs.
365	1085a	0.06	0.5	" " 29 "
355	1092	0.08	—	" " 29 "
360	1092	0.1	—	" " 14-16 "
360	1092	0.1	0.5	" " 22 "
350	1228	0.05	—	Lives
360	1228	0.06	—	Dead in 40 hrs.
360	1228	0.07	0.5	Lives

same was true of Sera 1192a, prepared with living cultures and filtrate respectively. Thus 2 cc. filtrate of (a) type or (b) type mixed with 2 cc. undiluted, homologous immune cows' sera both "living" and "filtrate," failed to moderate the reaction. In one calf (1159) the addition of 6 cc. "filtrate" serum did not check a severe reaction. The lungs of this calf 2 days after injection were still focally hemorrhagic and generally congested. If any mitigation of the toxic effects is to be produced by immune sera, it obviously will require prolonged immunization and large doses of the immune sera.

*Polyvalency of Immune Sera.*—The serological relationship of the

different strains of *B. coli* isolated from the ileum during scours or in normal condition was determined by using immune cow serum. When the minimum fatal dose was mixed with the serum a certain grouping was made possible. In Table IV two strains were obviously modified in their pathogenic action by the serum. In two others the same quantity was not sufficient to prevent death.

The overlapping of the protective action of sera from two cows treated with different strains is brought out in Table V. Where the

TABLE V.  
*Polyvalency of Immune Cow Serum.*

Strain	Minimum fatal dose	Protective dose of serum in 0.5 cc. or less		Culture character
		1127a	1192a	
	cc.	cc.	cc.	
223a	0.06-0.07	>0.5	0.5 or less	Saccharose + (motile)
1069a	0.5	>0.5	—	
1085a	0.04	0.2-0.3	>0.5	
1085b	1.0	0.5	—	
1092	0.1	0.3-0.4	>0.5	Saccharose + (motile)
1127a	0.2	0.1	—	
1127b	0.7	0.03	—	
1179	0.4	>1.5	—	
1189	0.5	>1.5	—	Saccharose +
1192a	0.04	0.3	0.05	
1192b	1.0	—	0.1	
1197	0.5	0.5	—	
1208	0.15	0.3	—	
1228	0.05	0.5	0.5 or less	
1358a	0.08	—	0.5 " "	

amount of serum needed is indicated as higher than the doses tried, there is of course the possibility that the serum has no effect whatever. Thus Strain 1179 probably belongs to an entirely different serological group. There is also the possibility that by prolonged treatment of the cow the serum might become protective in the doses used.

It is of interest to consider the relative neutralizing power of immune serum towards the (a) and (b) forms. In an early test of serum of Cow 1109 (*B. coli* 1127a), 0.1 cc. serum inhibited the surely

fatal dose of 0.2 cc. of the homologous strain. 0.03 cc. of the same serum inhibited the surely fatal dose of 0.7 cc. of the (b) mutant. A more detailed test was made with an immune serum of Cow 1231 (*B. coli* 1192a). The final tests are given in Table VI. Tests made with the same drawing of serum on the (a) form showed that the surely fatal dose of 0.04 cc. ( $1\frac{1}{3} \times \text{M.F.D.}$ ) was inhibited by 0.005 cc. serum.

There is thus very little difference in the protective power of the (a) serum on the surely fatal doses, 0.04 and 0.6 cc., of the (a) and (b) forms respectively.

*Non-Specific or Natural Resistance in Guinea Pigs.*—It has been stated that guinea pigs are capable of disposing of considerable num-

TABLE VI.  
*Immune Cow Serum 1192a and Culture B. coli 1192b.*

Date of test	Weight of guinea pig	Dose of culture	Dose of serum	Result
	gm.	cc.	cc.	
Feb. 4	370	0.5	—	Dead in 12 hrs.
" 4	360	0.6	—	" " 12 "
" 9	350	0.6	—	" " 15-16 "
" 9	355	0.6	0.005	Lives
" 9	330	0.6	0.02	"
" 11	345	0.6	—	Dead in 13 hrs.
" 11	355	0.6	0.0025	Lives

bers of colon bacteria even when of maximum virulence. This phenomenon may be ascribed to a natural immunity. It had been shown by R. Pfeiffer and Issaëff<sup>5</sup> that by preparing the abdominal cavity of guinea pigs by the injection of bouillon, salt solution, and the like, an increased resistance towards the cholera vibrio could be produced. The same is true when *B. coli* is used. When guinea pigs receive into the peritoneal cavity 2 cc. bouillon, the minimum fatal dose of *B. coli*, or even a larger dose, is readily borne when injected 2 days later. If the experiment is continued and the same dose injected 10 to 14 days later, the guinea pig may die. In other words, the animal has lost in resistance during this period even though the

<sup>5</sup> Pfeiffer, R., and Issaëff, Z. *Hyg. u. Infektionskrankh.*, 1894, xvii, 355.

loss in weight following the injection of the first fatal dose has been amply compensated. Repeated trials were made to eliminate any possible errors in the tests. All showed that at least a certain number of guinea pigs had not recovered their normal resistance. In Table VII a final test is given. Eight guinea pigs received 2 cc. bouillon into the peritoneal cavity. 2 days later their weights had all increased. Three of them received 0.02 cc., and three, 0.025 cc. culture fluid. 2 days previous 0.022 cc. was found fatal. 11 days later all eight received doses from 0.016 cc. to 0.022 cc. in amount. Of these, two having received 0.02 cc. died within 10 hours. It will

TABLE VII.

Original weight of guinea pig	All injected intraperitoneally, 2 cc. of sterile bouillon				Result	Weight 9 days after 2nd inoculation
	Injected 2 days later		Injected 11 days later			
	Weight	Dose of <i>B. coli</i> culture	Weight	Dose of <i>B. coli</i> culture.		
gm.	gm.	cc.	gm.	cc.		gm.
385	—	—	440	0.02	Lives	400
355	—	—	450	0.022	"	430
375	395	0.02	420	0.016	"	415
370	400	0.02	430	0.02	Dead in 10 hrs.	—
375	390	0.02	430	0.018	Lives	445
370	400	0.025	430	0.016	"	430
365	385	0.025	445	0.02	"	440
390	400	0.025	460	0.02	Dead in 10 hrs.	—

be noticed that 0.022 cc. did not kill a control; and that of the two that died, one had easily borne a dose one-fourth higher than the one which proved fatal. The other died following the same dose originally borne successfully. In this experiment all bouillon used for *B. coli* cultures was from the same lot.

Following an initial dose of *B. coli*, the recovered animals in a few instances have the spleen partly bound down by adhesions. The peritoneum recovers fully its original normal appearance. Permanent opacities and infiltrations are absent. The failure to resist the second dose does not appear to reside in any distinct anatomical defects or changes.

## DISCUSSION.

In attempting to formulate the mechanisms by which the guinea pig is injured as well as protected we have several phases of the work to assist us. The study of the effect of the filtrate or toxin on calves shows us that most, if not all, of the pathological effect of the bacillus may be accounted for by its toxic filtrate. The extreme dilatation of the capillary system followed by hemorrhage as found in calves described also its action on guinea pigs, in spite of the fact that this species appears to be relatively indifferent to the culture filtrates. The guinea pigs either die within 24 hours or appear lively after 12 hours. Very few die on the 2nd day. The offensive weapon, then, of *B. coli* appears to be its soluble, diffusible toxins. Another fact which favors the view of the toxin action of *B. coli* is the difference in virulence due to different lots of bouillon. With *B. coli* 1192a the minimum fatal dose increased 2 to  $2\frac{1}{2}$  times from one lot to another.

The offensive action of the guinea pig probably is not a neutralization of this toxic filtrate, for in calves we find that a relatively large amount of immune serum mixed with filtrate does not relieve the symptoms due to the toxin. The guinea pig probably protects itself by some stored natural protective substance which, if present in sufficient quantity, checks the bacteria. If not, the bacteria continue to multiply and toxin production goes on until death ensues. This substance is mobilized by the injection of non-specific substances such as bouillon, so that a minimum fatal dose is easily borne. On the other hand, it appears to be used up by the injection of cultures and not fully restored even after 10 to 14 days, because at this time a second fatal dose or even a smaller dose may be fatal. If specific immunity consists in an overproduction of this substance it follows that resistance can be induced in guinea pigs only very gradually. It is highly probable that this substance destroys the bacteria by lysis, as is known for the cholera vibrio. The cooperation of polymorphs in this phase is highly doubtful. The cells appear too late on the scene. The macrophages come still later, after the fate of the guinea pig has been settled.

To protect itself against this factor in the host, certain colon bacilli have developed a capsular substance. That this bacterial substance is primarily protective is suggested by its absence in mutants. They



retain the toxin-producing capacity, but lose, largely or wholly, the capsular, viscid substance and with it their specific resistance to agglutination and phagocytosis. Their virulence towards the guinea pig dropped in one culture to  $\frac{1}{3}$ , in several others to  $\frac{1}{15}$  of the original dose. This change is completed a few days after isolation on agar plates. That death is still caused by the mutants is accounted for by the still normal toxin production. The large minimum fatal dose of living bacilli of mutant type is necessary since this type is readily destroyed and must be present in excess at the start.

The injection of living cultures of *B. coli* of the (a) or viscid type

TABLE VIII.

	Bacterial activities	Host activities
<i>B. coli</i> (a) type (M.F.D.: 0.04 cc. or 0.025)	Toxin $\longrightarrow$ kills (offensive)	(Normal) antibody towards toxin, if any, increased very slowly or not at all by treatment
	Capsular substance (defensive)	$\longleftarrow$ (Normal) antibody greatly augmented by treatment (vaccina- tion, immunization)
<i>B. coli</i> <sub>2</sub> (b) type (M.F.D.: 1.0 cc. or 0.6)	Toxin $\longrightarrow$ kills (offensive)	$\longleftarrow$ Unless host overpowered by toxin at the start, bacteria killed readily by antibodies
	Capsular substance (present in traces or absent) (defensive)	

leads to the production of an immune serum which mixed with cultures and injected has a definite and potent protective action whereas normal cow serum has none in evidence in the technique used. When such normal serum is injected in quantities up to 2 cc., with cultures, death is not retarded. As stated above, the immune serum is not antitoxic. It probably cooperates with the normal forces of the guinea pig in suppressing multiplication and destroying the bacteria, since only a few survive in the peritoneal cavity after 24 hours. The interrelations of the several host-parasite activities, as suggested by the data given, are presented in Table VIII.

The presence of normal, antibacterial forces in the body and the production of highly specific antibodies by the repeated injection of

cultures have always been more or less incompatible factors in theories of immunity. The results reported in these papers offer the following hypothesis towards harmonizing these factors. The peritoneal cavity of the guinea pig and its walls (endothelium?) contain some substances normally which destroy the injected bacteria either by removing a protecting, viscid envelope and so exposing the organism to lysis, or by checking multiplication in one or more ways such as making the bacterial membrane more permeable to lytic factors or less permeable to nutritive elements. These substances are mobilized by the preliminary injection of bouillon. When some animal host is repeatedly treated with cultures, some one of these protective factors is highly developed in accordance with the capacities of the host. This gives the immune serum a one-sided, more or less specific effect, since the bacteria are suppressed in a certain way only.

According to this hypothesis, each host might furnish a serum of slightly different antibacterial activity towards the same microorganism according to the specific bacterial factor most easily handled by such host. It is conceivable that if the toxin of *B. coli* is antigenic, some host may protect itself chiefly by developing an antitoxin. The reason for serum specificity towards different biological groups of bacteria may be explained in a similar way, in that the bacteria themselves may present several widely different points of attack. Natural immunity according to this theory is simply a reservoir of numerous natural possibilities to be stimulated according to the different patterns presented by the microbes and the different capacities of the hosts for overdeveloping the individual contents of this reservoir. In particular the outer functional element, the ectoplasm, capsule, membrane, of the parasite, whatever it may be called, seems to stand out as the most important of the bacterial factors to be met by the host. If there is an active secretory function throwing off protective material, this, becoming diffused through the system of the host, may bring many cell territories to cooperate in increasing the output of protective antibodies. Immunity may come quickly and permanently, or else the microorganism gains the upper hand equally rapidly as in anthrax. The less the outer functional element is concerned in secreting and excreting protective substances, and the more it is concerned in transforming itself into a

locally protective envelope difficult to attack, the more the immunizing process becomes localized, and the slower the multiplication of the bacteria and the more difficult the acquisition of general immunity becomes.

The paratyphoid group, probably an offshoot of the colon group, may serve to illustrate this hypothesis. The paratyphoid bacillus produces a toxin which has similar effects on calves. It has no original capsular or viscid substance. None of the group yields any satisfactory antisera such as are readily produced by the frequently capsulated bipolar pneumococcus and colon groups. These invasive forms are however able to reach sensitive tissues and to produce their toxin in the close vicinity of highly sensitive cells. What this may mean is illustrated by *B. coli*. The toxins of this group introduced into the subcutis have little systemic effect. Brought in contact with the pulmonary epithelium artificially, as in intravenous injections, a very small amount produces profound effects.

#### CONCLUSION.

The interrelations between bacterial toxins, bacterial capsular (mucoid, viscid) substance, and certain normal protective factors in the guinea pig are studied with the aid of bacterial mutants and immune serum, and the results formulated in an hypothesis.

# STUDIES ON PATHOGENIC *B. COLI* FROM BOVINE SOURCES.

## IV. A BIOCHEMICAL STUDY OF THE CAPSULAR SUBSTANCE.

By DOROTHEA E. SMITH, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, March 18, 1927.)

The carbohydrate gums which have been obtained from bacteria in sufficient quantity for chemical study are few in number. The early workers were concerned with capsular material as such, and the possibility of its precipitation by homologous immune serum had not been thought of. A bibliography and review of the literature are to be found in the papers of Heidelberger and Avery (1), or of Heidelberger (2).

Preisz (3) studied in detail the rôle of capsule and capsular material in infectious disease. He made preparations of capsular material in considerable amount. He believed it to be a mucin and stated that it gave no reducing sugar reaction. Whether the substance was prepared from virulent or avirulent strains, it removed from sera the factors responsible for lysis or the killing of anthrax bacilli (page 399). But he did not consider this a specific reaction, because similar material from cultures of organisms resembling *B. anthracis* also removed bactericidal substances from antianthrax sera *in vitro*. He thought that in the last stages of infection in the rabbit, capsular substance was present in blood and exudate in solution, neutralizing the antibody. He maintained that there was throughout a parallelism between virulence and the possession of a capsule.

With observations from studies such as this two lines of evidence converged towards and merged into the present concept of a polysaccharide reacting specifically with homologous serum, responsible for type specificity, but not eliciting antibody formation, and in all probability connected with the possession of a capsule and virulence in certain cases.

1. In 1921 Zinsser (4) called attention to the immunological importance of the non-protein constituents of bacterial cells. This frac-

tion which reacts with specific serum antibodies was termed "residue" in order not to imply too accurate an idea of its chemical structure. He believed this to represent the so called "haptene" of Landsteiner (5), for Landsteiner in his studies upon chemical modifications of precipitable antigens foretold the probable existence of materials which would react with antibodies without themselves being capable of inducing antibody formation when injected into the animal body. These conditions Zinsser's "residue" fulfilled, and the suggestion was made that they represented the haptophore group split off from the antigen as a whole and molecularly too small to induce antibody formation. It was also stated that this "residue" was analogous to the substances previously found by Cole (6) and by Dochez and Avery (7) in the blood and urine of typhoid and pneumonia patients.

2. The mass of evidence presented in the fundamental papers of Avery and Heidelberger (2) and Heidelberger and Avery (2) leaves us without grounds for doubting that it is a protein-free preparation that reacts specifically with homologous precipitating antibody and is responsible for type specificity. Very marked chemical differences were found correlated with serological specificity.

In the work of Mueller (8) and of Heidelberger, Goebel, and Avery (9) it was suggested that the "residue" or soluble specific substance is either identical with or connected with the capsule. But a question very naturally arises, why then is it possible to obtain "residue" from such apparently uncapsulated organisms as the tubercle bacillus, the staphylococcus, the meningococcus, and the influenza bacillus (10), as well as from the capsulated types of pneumococcus and the Friedländer bacillus? Is the capsule due to an increased production of a substance always present in the bacterial cell, a substance capable of reacting with antibody, but not of eliciting its formation on injection? Such a substance might represent the "haptophore" group of Ehrlich, and meet the conditions of the "haptene" prophesied by Landsteiner. Another question that naturally arises is the relation between capsular substance and virulence. A brief survey of the literature at once shows us that the possession of a capsule is almost universally regarded as increasing the virulence of an organism. This might be brought about in two ways. In the first place the capsular substance might act as a morphological factor, not dissolving away from

the organism, and insulating it from antibody action. Secondly, it might protect the organism by reacting in solution, at some distance from the bacterial cell, with antibody.

#### EXPERIMENTAL.

The possession of a number of mutating cultures of *B. coli* described by Theobald Smith and associates (11-13) offered an opportunity to look for the answers to the above questions in the case of this one species. It should be possible to find out if more precipitable carbohydrate is obtainable from the capsulated than from the non-capsulated strain from equal areas of culture surface, what its probable relation to the greater virulence of the capsulated strain may be, as well as something about its chemical properties.

A consideration of the literature leads us to expect that a capsule would increase the virulence of an organism. This is, therefore, a very unique opportunity to obtain a quantitative estimate of the extent to which it enhances virulence. We think we are justified in excluding other factors, because the organisms are so alike. One, called the (b) form, is derived as a mutant from a colony of the other, called (a). Precipitation in the culture filtrate of either one is caused by the serum of the other, as well as by its own serum, as will be shown later. Filtrates of bouillon cultures, 24 to 48 hours old, of both (a) and (b) forms when injected into calves exert marked toxicity (11), but guinea pigs are only slightly sensitive.

These cultures of *B. coli* also afford an opportunity to test out the suggestion that "residue" or soluble specific substance is either identical with, or connected with, the capsule; because, if a certain substance were found to be present in extracts of the capsulated bacillus, and either not present at all or present in very small amounts in similar extracts of the mutant, it would be good evidence that it was indeed the material of the capsule. Of course it does not prove that there are not small amounts of other substances present, such as mucin. In the case of an alkaline extract of an extremely viscid strain of Friedländer's bacillus previously studied by the writer, there was present a large amount of substance or substances containing very little nitrogen in addition to the specifically active carbohydrate.

But in the case of the capsule of the colon strain there was nothing similar, at least not in appreciable amount

In what follows, the special strain of *B. coli*, 1192a, and its mutant 1192b, studied by T. Smith (11-13) was used throughout. In preliminary experiments, in which an alkaline extract of the bacteria was centrifuged and the supernatant precipitated with 2 volumes of 95 per cent alcohol, the precipitate, in the case of the viscid strain, was found to show a white ring very promptly at a dilution of 1 part in 100,000 by weight, with homologous serum. The mutant, non-viscid strain yielded a much less active precipitate, which gave a similar test more slowly and only up to a dilution of 1:1000 by weight, with either serum. It would seem therefore that in one case there was about a hundred times as much precipitable substance as in the other, so it seemed worth while to try to isolate the substance.

*Preparation of Soluble Specific or Capsular Substance.*—In all about 90 ordinary tin pie plates, each covered with a tin plate of larger size, were poured and inoculated. The ordinary stock veal infusion agar kept on hand in the laboratory was used, with the addition of 0.1 per cent dextrose just before pouring. The agar surfaces were very liberally inoculated with a suspension from 24 hour agar slants in normal saline. After 2 days' incubation, the growth was removed in distilled water (14). In this condition it was still extremely viscous. The bacterial emulsion was diluted to a volume of 500 cc., treated with about 75 cc. of 10 per cent KOH, warmed to 70°, and kept at that temperature about 30 minutes. After neutralizing and making slightly alkaline to litmus, adding about 10 gm. of sodium acetate and 150 cc. of 95 per cent alcohol, a clear supernatant was obtained after centrifugation and the sediment of bacterial bodies rejected. The substance in the supernatant was precipitated on adding 95 per cent alcohol up to 1.2 volumes. It was partially purified by repeated solution and precipitation with 1.2 volumes of 95 per cent alcohol, added drop by drop with mechanical stirring, in the presence of plenty of electrolyte in the form of sodium acetate, usually from solution distinctly alkaline to litmus. Precipitation from acid solution was also employed. The discarded supernatant contained much orange-brown material and very little specific substance as determined by the precipitin test. Precipitation with alcohol was continued 5 to 7 times, usually until the supernatant was practically colorless. The final precipitate, which unlike similar Friedländer material dissolves readily in warm distilled water, was put through a Berkefeld filter, after sufficient dilution, if still turbid. After concentration to convenient volume and reprecipitation with alcohol, it was washed with alcohol in increasing concentration and dried *in vacuo* over sulfuric acid. The yield from 90 plates was somewhat over 2.3 gm. It was active with homologous serum when diluted to 1 part in 2 millions, using the Fornet-Müller ring test.

*Description and Analysis.*<sup>1</sup>—The biuret test was negative. There was no precipitate (in  $\frac{1}{2}$  per cent solutions) with tannic acid, phosphotungstic acid, Esbach's reagent, 5 per cent copper sulfate solution, 10 per cent uranyl acetate solution, or saturated barium hydroxide solution. There was no color with iodine. The Molisch test was strongly positive. But with 10 per cent basic lead acetate there was heavy precipitation and with 10 per cent ferric chloride a fairly heavy precipitate soluble in excess of the reagent.

The white, fluffy powder is readily soluble in hot water. In even a 1 per cent solution it is viscous and opalescent, somewhat resembling starch. It is not stringy, like the original growth on agar. It is very readily hydrolyzed by boiling with acid, and the hydrolysate reduces Fehling's solution, showing that a reducing sugar is present. It gives a slight naphthoresorcinol test for glucuronic acid and a slight test with orcinol, indicating the presence of glucuronic acid, but too faint for a pentose. There is a faint turbidity on boiling with barium hydroxide which is not removed by the addition of hydrochloric acid, thus indicating the presence of a hydrolyzable sulfuric acid. Very likely this is due to an impurity.

The substance, on drying in an Abderhalden dryer at 100°, lost 11.34 per cent of water. The elementary analysis, which was very kindly done by Dr. Elek, was as follows:

Ash 9.24 per cent —

C = 42.26 per cent

H = 5.82 " "

S = 0.6 " "

N = 0.6 " " (my own figure, micro-Kjeldahl,  
on 10 mg. portion)

Calculated on ash-free basis —

C = 46.56 per cent

H = 6.41 " "

For  $(C_6H_{10}O_5)_x$  —

C = 44.4 per cent

H = 6.2 " "

So the carbohydrate nature of the substance is evident.

1 gm. of powder was used in the preparation of an osazone. It was hydrolyzed by boiling in 10 cc. of 2 per cent hydrochloric acid

<sup>1</sup> The writer was greatly assisted by Dr. P. A. Levene in this portion of the work.



under return condenser. The reaction was carefully followed by observing the rotation and the reducing sugar present in the solution from time to time.

Rotation		Reducing power equivalent to glucose
Initial	0.05	—
After $\frac{1}{2}$ hr.	0.06	—
“ $1\frac{1}{2}$ hrs.	0.06	800 mg. (about 80 per cent)
“ $5\frac{1}{2}$ “	0.08	785 “

Since the values in the above table showed no significant change, heating was stopped, and the hydrolysis mixture neutralized with sodium hydroxide till only slightly acid to litmus. It was filtered and concentrated under reduced pressure to 60 cc. To obtain a phenylosazone it was warmed on the water bath with 2 gm. of free phenylhydrazine dissolved in glacial acetic acid. After 45 minutes on the water bath, it was heated a trifle more and immediately filtered through a folded filter. To this filtrate a second time phenylhydrazine in glacial acetic acid, to the amount of 1 gm., was added and the solution kept on the water bath as long as an osazone was forming. This was again filtered off, and a third precipitate of osazone obtained from the filtrate after standing about 1 hour more. The first lot of osazone was kept separate, but the second and third, which had a similar appearance, were combined. Each precipitate was taken up in methyl alcohol to remove adhering impurities, so that the final osazones were free from oil drops and consisted of long crystalline needles or rosettes.

The melting point of the first was 196°C. The second sintered at 180° and decomposed at 195°. Neither showed optical rotation. The second was analyzed for nitrogen, with the following results: From 0.0620 gm. substance was obtained 8.30 cc. nitrogen at 763.3 mm. Hg and 22°C. This is equivalent to 15.55 per cent nitrogen. (Calculated for  $C_{18}H_{22}O_4N_4$ , N = 15.63 per cent.)

The fact that the observed rotation of the hydrolysate was very low for the reducing power, and that the osazone seemed to be entirely inactive optically, makes it seem likely that the hydrolysis mixture contained at least two hexoses, one dextro- and the other levorotatory. The osazones were not separable, or they may have been identical. From these facts it seems established that the substance obtained

from the colon bacillus belongs in the class of carbohydrates, as shown by the elementary analysis, that it contains glucuronic acid, and hexoses. The total reducing power of the sugars obtained on hydrolysis calculated as glucose was equivalent to 80 per cent of the weight of the substance. The substance thus differs from all the others reported.

*Attempt to Relate the Specific Carbohydrate Obtained from the Capsulated Bacillus to Its Greater Virulence.*—The question naturally arose<sup>2</sup> as to whether it were possible to find out how the capsular material acts to increase virulence. Whether it may function as a morphological capsule, or partly dissolve and thus act at a distance by neutralizing the antibody in the blood of the host.

TABLE I.

Guinea pig No.	Weight	Culture	Extract	Result
	<i>gm.</i>	<i>cc.</i>	<i>mg.</i>	
1	355	0.3	—	Lives
2	350	0.6	—	"
3	350	0.9	—	"
4	360	1.2	—	Dead in 22 hrs.
5	355	0.3	10	Lives
6	350	0.6	10	"
7	350	0.9	10	Dead in 10 hrs.

It seems, from a consideration of the following observations, that in the case of the viscid strain the substance must be acting to a large extent as a closely adherent protecting layer, probably permitting multiplication of the organisms before they can be destroyed. We know from the papers by Smith and associates (11-13) on these mutants that capsulated strains are practically not phagocytized at all. If capsular substance is of significance in the production of virulence it may very likely be in connection with non-phagocytability.

A few experiments were made on guinea pigs to see if the substance extracted from cultures as a carbohydrate had any influence on the course of the disease produced by *B. coli*. The mutant (b) was tried

<sup>2</sup> For suggestions at this point the writer is particularly indebted to Dr. Hans Zinsser and Dr. J. H. Mueller.

first. Table I illustrates the procedure for testing any increase in virulence due to admixture of the living 24 hour bouillon culture with the extract. This was ground up in normal saline. The injection was made into the peritoneal cavity of guinea pigs.

An apparent increase in virulence may be deduced from the table. Several other tests yielded similar data. The (a) form was tried next to see whether it also might be favored by the preparation. 5 mg. of the preparation in normal saline were mixed with graded amounts of the culture and the mixture injected into the peritoneal cavity. The minimum fatal dose was definitely lowered. When, as controls, 5

TABLE II.

1192a and 1192b grown 24 hrs. in flasks containing 75 cc. of veal bouillon.

Serum of rabbit injected with strain	Capsulated strain, 1192a			Mutant, 1192b		
	Filtrate not diluted	Diluted 1/2	Diluted 1/4	Filtrate not diluted	Diluted 1/2	Diluted 1/4
(a)	+	Tr.	0	+	+	Tr.
(b)	+	0 (?)	0	+	0 (?)	0

1192a and 1192b grown 48 hrs. in exactly similar conditions.

(a)	+	?	0	+	+	Tr.
(b)	+	0	—	+	0	—

1192a and 1192b grown 3 days in exactly similar conditions.

(a)	+	+	0	++	+	Tr.
(b)	+	+	0	++	+	0 (?)

\* + means a ring that can be seen distinctly. None of these rings were heavy.

mg. of gum tragacanth, and a feebly turbid homogeneous suspension of aleuronat, probably less than 5 mg., were injected with *B. coli*, the same increase in virulence was observed.<sup>3</sup> This method of demonstrating the possible relation of the carbohydrate to virulence was not pursued any farther, since it was evident that some other method would have to be worked out, eliminating mere injury to the peritoneum. The protocols relating to the test with the (a) form are therefore omitted.

<sup>3</sup> See also Benians (15).

It is to be noted that the amount of precipitable substance found in filtrates of early cultures is actually very small, both in the (a) and the (b) forms. This is brought out in Table II.

The two rabbits yielding the highest titer serum, Nos. 1 and 2, had been given intraperitoneal injections, 5 days apart, of increasing amounts of killed growth from 24 hour agar slants emulsified in normal saline. It is impossible to clear the culture of the capsulated strain by centrifuging. An attempt was made to see if any rings could be distinguished in a dilution of 1:8 to eliminate turbidity, but there was not enough precipitable substance to give a reaction at this dilution. So recourse to filtration through a small Berkefeld was necessary. Saline was always passed through first and tested to insure absence of anything giving a ring with the most potent serum (a). Then half the 75 cc. of culture fluid was passed through and rejected, since precipitable substance might be adsorbed by the filter at first. Inasmuch as all the material of all preparations made had been put through Berkefeld V's or N's and there was no trouble with adsorption, the precaution of using only the last part of the filtrate would seem sufficient.

#### DISCUSSION.

The condition presented by *B. coli* (a) and (b) was quite different from that found by Dochez and Avery (7) when studying the pneumococcus. During the early stages of vigorous growth this organism forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the blood and urine. The writers could not demonstrate that this substance was responsible for the intoxication occurring in lobar pneumonia, but they were able to state, after the study of 112 cases, that if large amounts were excreted, the outcome was usually fatal. The table on page 479 of Dochez and Avery's paper is particularly instructive. This is from the study of a flask inoculated with an organism of Type III, which forms somewhat more precipitable substance than I or II. After only 4 hours, the undiluted filtrate showed heavy flocculation with homologous serum. A 24 hour culture showed a heavy precipitate at a dilution of 1:30. A trace was recorded at a dilution of

1:120. Evidently the material diffuses out into the culture fluid in considerable amounts.

When we consider the capsulated *B. coli*, we find an entirely different state of things. As shown in the table, there was not a trace of precipitin reaction visible in the case of a 24 hour culture at a dilution of 1:4, nor was there in that of a 3 day culture. When undiluted, there was a good, but not a particularly heavy reaction, even with a precipitating serum of very high titer. Evidently in this case the precipitable substance does not dissolve out into the medium during the growth period. The amount of specific carbohydrate capable of reacting with homologous serum in a 1 to 3 day culture of either (a) or (b) is practically nil.

There remains the question of the relative amounts of precipitable material present in the two strains, the one capsulated and the other not, for this ratio is perhaps at this time the best chemical evidence obtainable of a relation between capsule and soluble specific substance. More crude material is obtainable from equal areas, for example, the surface of ten pie plates in the case of the capsulated strain. No count of the relative numbers of organisms involved has been attempted, but from the uncapsulated strain only about two-thirds as much crude material can be obtained per unit of culture surface. And this material is about 100 times *less active* with high titer precipitating serum (from either strain) than is similar material from the capsulated strain. No attempt has been made to prepare or purify a large amount of the "residue" from the mutant.

The simplest assumption is therefore that suggested by Mueller (8) and Heidelberger, Goebel, and Avery (9), that capsular material and soluble specific substance are identical. In the case of a morphological capsule, the specifically precipitable substance or "residue" probably is produced in much larger quantity and located peripherally. Other substances, such as bacterial mucins, may also take part in the capsule formation. In the case of the capsulated *B. coli*, there was no indication of more than a trace of mucin. From the ease with which the material can be obtained in relatively pure condition, it is very unlikely that much is present. On the other hand, in the preparation of specific carbohydrates from a peculiarly viscid strain of Friedländer's bacillus (unpublished) there was obviously a *very*

large admixture of an impurity that was probably mucin, or something similar.

The substance obtained from the capsulated strain of the colon bacillus is not identical with any specific carbohydrate thus far already described. It is composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is slight. It is precipitable by basic lead acetate and ferric chloride. It is rather lighter in weight and more readily soluble in distilled water than are some similar substances prepared by the writer from the Friedländer bacillus. The presence of glucuronic acid is indicated. Some evidence for a relation between capsule and specific substance was obtained. From equivalent areas of growth on agar in the case of the mutant about two-thirds as much material, as in the case of the viscid strain, precipitable with 1 volume of alcohol, can be obtained. This material, obviously very impure, is about 100 times *less* active with homologous serum than similar material from the capsulated strain. Although this cannot be interpreted as proving that the capsulated organism contains 100 times as much specific carbohydrate as the mutant, it is a very significant difference.

The specific substance of the capsulated colon strain does not diffuse out into the fluid culture media, as does that of the pneumococcus. There is so little present in the filtrate of a 24 hour culture, such as used in the original virulence tests, that it indicates that the substance functions distinctly as a capsule, a protecting layer, rather than in solution. Attempts to recombine the extract with living bacilli in testing virulence proved unsatisfactory, since non-specific additions in minute amounts also reduced the resistance of the peritoneal cavity of the guinea pigs.

#### SUMMARY AND CONCLUSIONS.

1. The soluble specific substance obtained from a capsulated strain of *B. coli* is not identical with any specific substance heretofore described. It is a carbohydrate, composed of 80 per cent of hexose, probably partly of dextro- and partly of levorotatory sugar, since the rotation of the hydrolysate is low. Glucuronic acid is probably present in the molecule.

2. Crude "residue" or specific substance obtained from the unencapsulated mutant was about 100 times less active with homologous serum than similar material from the encapsulated strain. This supports the view that capsular substance and soluble specific substance are the same. In cases where there is a well marked capsule, the specific substance is probably produced in greater amount and located peripherally.

3. Capsular substance is probably significant for virulence when functioning as a morphological capsule. It is present in filtrates of young culture only in very small amounts.

#### BIBLIOGRAPHY.

1. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73.
2. Heidelberger, M., *Physiol. Rev.*, 1927, vii, 107.
3. Preisz, H., *Centr. Bakt., I. Abt., Orig.*, 1909, xlix, 341.
4. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
5. Landsteiner, K., *Biochem. Z.*, 1918-19, xciii, 106.
6. Cole, R., *J. Exp. Med.*, 1917, xxvi, 453.
7. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
8. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924-25, xxii, 373.
9. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 701.
10. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
11. Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, xlvi, 123.
12. Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlvi, 133.
13. Smith, T., *J. Exp. Med.*, 1927, xlvi, 141.
14. Toennissen, E., *Centr. Bakt., I. Abt., Orig.*, 1920-21, lxxxv, 225.
15. Benians, T. H. C., *Brit. J. Exp. Path.*, 1924, v, 123.

# THE SURFACE COMPOSITION OF THE TUBERCLE BACILLUS AND OTHER ACID-FAST BACTERIA.

BY STUART MUDD, M.D., AND EMILY B. H. MUDD.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

(Received for publication, April 15, 1927.)

The tubercle bacillus and other acid-fast bacteria are remarkable for their high lipin content, for their difficult but tenacious staining properties, and for their resistance to the ordinary destructive agencies of the body. It seemed natural in correlating these properties to postulate for the tubercle bacillus a difficultly permeable lipoid envelope<sup>1</sup> (Ehrlich) to which its tinctorial and biological peculiarities were ascribed. So simple a conception of the surface of the tubercle bacillus has become untenable in the light of recent investigations. Evidence will be presented in the present communications to indicate that the surfaces of the acid-fast bacteria are complex, containing at least lipin and protein, and quite likely also carbohydrate components; that these surfaces are profoundly modified as the result of interaction with serum; and that the acid-fast staining property is independent of the state of the surface.

## *Evidence for the Existence of Lipin in the Surface.*

The most direct evidence for the presence of lipin in the surface of the acid-fast bacteria is the striking and characteristic ease with which they are wetted by oil or fatty acid. These microorganisms have been studied in oil-water interfaces in the manner already described for bacteria and blood cells,<sup>2</sup> and observations on more than 1000 such film preparations with modified and unmodified acid-fast bacteria have been recorded. Normal acid-fast bacteria grown on glycerol agar slants and suspended in 0.8 per cent sodium chloride

<sup>1</sup> Ehrlich, P., *Deutsch. med. Woch.*, 1882, viii, 269.

<sup>2</sup> Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633, 647; 1926, xliii, 127. Mudd, S., *Proc. Soc. Exp. Biol. and Med.*, 1925-1926, xxiii, 569.



solution, certain mammalian tubercle bacilli excepted, pass spontaneously through the interface into the test oil, tricaprylin (Kahlbaum).

This typical spontaneous passage through the interface into oil has been observed repeatedly with the strains listed below.

*M. tuberculosis*: Bovine III, Bovine C, Bovine 816, and Bovine 960 (816 and 960 are isolations from tuberculous laboratory animals and probably the same strain as Bovine C); *M. avium*: Egret, Avian III, "lepra bacillus" of Kedrowsky,<sup>3</sup> homogeneous culture of Arloing;<sup>3</sup> "tubercle bacilli" of cold blooded animals: *M. chelonci*, *M. ranæ*, *M. marinum*;<sup>4</sup> saprophytes: *M. smegmatis*, "pseudoperlsucht"<sup>5</sup> bacillus, "milk" bacillus.

The behavior described for acid-fast bacteria is in contrast to that of other bacteria which are very stable in the interface,<sup>2</sup> and to blood cells<sup>2</sup> and spermatozoa,<sup>6</sup> all of which possess more or less stability in the oil-water interface and offer resistance to passage from the interface into the oil phase.

The bacteria were removed from the surface of glycerol agar slants with platinum wire and were suspended directly or with grinding in 0.8 per cent sodium chloride solution. They were then washed in 0.8 per cent sodium chloride and studied against an oil immediately, or after standing overnight or longer in sodium chloride solution. As the slowly advancing oil phase touched the margin of a clump of bacilli suspended or sedimented in the sodium chloride phase, the interface ran forward locally, the oil swiftly wetting the bacteria. If the bacilli were not too strongly coherent the clumps were dispersed explosively by the tension along the interface, and the bacteria flung violently into the oil phase. Such violent dispersion was characteristic of bacteria which can be suspended with comparative ease, *i.e.*, avian tubercle bacilli, the "tubercle bacilli" of cold blooded animals, "leprosy bacilli" of Kedrowsky, and an atypical bovine strain, Bovine III.<sup>7,8</sup> Loose clumps formed by sedimentation from an even bacterial suspension were similarly dispersed by the interfacial tension and flung into the oil. Suspensions of a virulent bovine tubercle bacillus, Bovine C,<sup>8</sup> which can only be made

---

<sup>3</sup> Furth, J., *J. Immunol.*, 1926, xii, 273.

<sup>4</sup> Aronson, J. D., *J. Infect. Dis.*, 1926, xxxix, 315.

<sup>5</sup> Incorrectly referred to as "*M. pseudotuberculosis*" in Reference 3 and in *J. Immunol.*, 1927, xiii, 115.

<sup>6</sup> Mudd, S., and Mudd, E. B. H., *Biochem. Z.*, in press.

<sup>7</sup> Smith, Theobald, *J. Med. Research*, 1913, xxviii, 91.

<sup>8</sup> Mudd, S., and Furth, J., *J. Immunol.*, 1927, xiii, 369.

with the aid of grinding, usually contain clumps so coherent as not to be dispersed by the interfacial stresses; the interface usually runs forward on such clumps and they pass entire into the oil.

As already indicated the preparations are ordinarily so made that the oil encroaches on the aqueous part of the film; the interface thus typically overtakes the bacteria suspended in the aqueous phase. Occasionally, however, the interface retraces its course for a time, thus overtaking bacteria which have already passed into the oil. Such bacteria do not pass back through the interface into the aqueous phase. On the contrary they are swept along before the retreating interface or may glide up or down in the interface. The oil-saline phase boundary is thus irreciprocally pervious to acid-fast bacteria; it permits spontaneous passage from saline to oil but not from oil to saline solution.

The four strains of human tubercle bacillus thus far studied, and one atypical bovine strain (B. C. G.), have differed from other acid-fast bacteria in not passing spontaneously into tricaprylin. These are the human strains H 37 (slightly virulent),<sup>8</sup> R 1 (avirulent),<sup>8</sup> Koch (avirulent),<sup>8</sup> P 15 B (highly virulent),<sup>9</sup> and the Pasteur Institute vaccine strain B. C. G.<sup>10</sup> All these strains have shown stability in the oil-saline interface; the degree of stability, however, is slight and not at all comparable to that of ordinary bacteria. Whether or not the slightly lesser oil miscibility<sup>11</sup> observed for these four human strains as compared to other acid-fast bacteria (B. C. G. excepted) is a characteristic property of the human type of tubercle bacillus we do not know. It is suggestive in this connection, however, that chemical evidence has seemed to indicate a higher wax but *lower fat* (*i.e.*, saponifiable lipin) content for human tubercle bacilli than for other acid-fast bacteria.<sup>12</sup>

<sup>8</sup> Perla, D., *J. Exp. Med.*, 1927, xlv, 215.

<sup>10</sup> Calmette, A., Guérin, C., Nègre, L., and Boquet, A., *Ann. Inst. Pasteur*, 1926, xl, 89.

<sup>11</sup> By "oil miscibility" in these communications we mean more precisely the property of being readily wet by oil.

<sup>12</sup> Frouin, A., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 606. Long, E. R., and Campbell, L. K., *Am. Rev. Tuberc.*, 1922-23, vi, 636. Wells, H. G., De Witt, L. M., and Long, E. R., *Chemistry of tuberculosis*, Baltimore, 1923, Chapter i.

Against tributyrin or triolein and several other oils<sup>2</sup> the acid-fast bacteria have possessed slight stability in the interface. This was true also of a sample of tricaprylin from another source than Kahlbaum. This tricaprylin was not optically clean by dark-field illumination, however, as Kahlbaum's has been. With no oil was the degree of stability of the acid-fast bacteria comparable to that of ordinary bacteria.

The surfaces of acid-fast bacteria grown on other media than glycerol agar slants have not been studied with any thoroughness. A culture of *M. chelonci* and one of a "milk" bacillus on Long's<sup>13</sup> synthetic liquid medium, and cultures of *M. ranæ* and *M. chelonci* on glycerol bouillon, were found to pass spontaneously into tricaprylin in typical fashion. An occasional experiment has shown, however, that impurities in liquid menstrua may sometimes coat acid-fast bacteria, causing change of surface properties simulating those produced by sera (see paper following).

### *The Mechanism of Acid-Fast Staining.*

Corroborative evidence of the existence of lipin in the surface of the acid-fast bacteria is furnished by the fact that the oil miscibility of the surface may be greatly decreased by extraction with alcohol. Tubercle bacilli (Strain Bovine III) were extracted with 95 per cent ethyl alcohol in a Soxhlet from 3 to 4 weeks by Dr. J. D. Aronson. Suspended in salt solution and studied with tricaprylin these extracted bacilli were highly stable in the interface, accumulated there in great numbers, and passed into the oil only with the expenditure of much mechanical work. Some "milk" bacilli were similarly extracted for 2 weeks and became stable in the interface. Both the extracted tubercle and "milk" bacilli retained their acid-fast staining properties. It is evident then that acid fastness does not depend upon the integrity of the surface membrane as originally postulated by Ehrlich.<sup>1</sup> A theory consonant with known facts has been suggested by Long.<sup>14</sup>

The surface lipin material can furthermore be coated over and the bacterial surface rendered oil-immiscible by sensitization with serum (see paper following). Such sensitized bacteria have shown normal acid-fast staining.

<sup>13</sup> Long, E. R., *Tubercle*, 1924, vi, 132.

<sup>14</sup> Wells, H. G., De Witt, L. M., and Long, E. R., *Chemistry of tuberculosis*, Baltimore, 1923, 101.

*Evidence for the Existence of Protein in the Surface.*

Evidence for the existence of protein in the surface has chiefly been derived by Freund<sup>15</sup> from study of the cataphoresis and agglutination of tubercle bacilli and their lipid and protein components. Both cataphoresis and agglutination in any given circumstances are conditioned by the cell surface. Freund found that the behavior of the whole bacilli in several important respects resembled the behavior of extracted or "defatted" bacilli and that of protein extracts rather than that of the lipid extracts. The isoelectric points found, for instance, were:

Whole tubercle bacilli, pH 3.0; defatted tubercle bacilli, pH 2.8; protein precipitated from watery extract, pH 2.8; lipid prepared from alcoholic extract, pH 1.5; lipid prepared from ether extract, pH 1.0.

The agglutination of the whole and defatted bacteria by electrolytes, moreover, resembled that of suspended protein and Gram-negative bacteria in that the higher concentrations of salts stabilized the suspension; the same salt concentrations did not prevent but indeed caused agglutination of the lipid particles.<sup>15</sup>

The existence of the group of human tubercle bacilli above described as differing in their surface-wetting properties from other typical acid-fast bacteria favors, although it of course does not prove, the conception of the acid-fast bacterial surface as complex. The most probable explanation of this difference is a quantitative difference between the two groups in the amount of lipin and protein or other polar component of the bacterial surface.

Dr. Freund was originally inclined to regard the surface of tubercle bacilli as entirely protein,<sup>15</sup> and the writer as essentially lipid. It is probably significant that Freund's cataphoresis experiments were performed with tubercle bacilli of human type. Human bacilli as already indicated have shown wetting properties differing less than those of other acid-fast bacteria from the properties of protein particles. Freund<sup>16</sup> and the writer are now agreed in regarding the surface of the acid-fast bacteria as complex.

A carbohydrate gum has been isolated from broth filtrates of

<sup>15</sup> Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124.

<sup>16</sup> Freund, J., personal communication.

human tubercle bacilli by Mueller<sup>17</sup> and by Laidlaw and Dudley from the bacilli themselves.<sup>18</sup> This carbohydrate in high dilution gives a specific precipitate with immune serum. It is known that the analogous carbohydrate haptenes in the pneumococci are components of the capsule.<sup>19</sup> It seems improbable that the carbohydrate hapten of the tubercle bacillus should be absent from the bacterial surface. Direct evidence bearing on this point is, so far as we know, however, entirely lacking.

#### SUMMARY.

Acid-fast bacteria in the boundary surface between salt solution and a test oil (tricaprylin) are spontaneously wet and enveloped by the oil. This behavior contrasts with that of all other cells studied by the interfacial tension method.

Four strains of human tubercle bacillus and an atypical bovine strain are an exception to the first statement above. These have possessed stability in the saline-oil interface; this stability is slight, however, and not comparable with that of non-acid-fast bacteria.

Acid-fast bacteria subjected to prolonged extraction with alcohol show resistance to wetting by oil comparable to that of non-acid-fast bacteria. These "defatted" bacteria nevertheless retain their acid-fast staining properties. Acid fastness cannot then depend on the integrity of a surface membrane.

Study of the cataphoresis of acid-fast bacteria by Freund has rendered the presence of protein in the surface highly probable. We are forced then to regard the surface of acid-fast bacteria as complex, containing at least lipid and protein. Not improbably also carbohydrate is present.

<sup>17</sup> Mueller, J. H., *J. Exp. Med.*, 1926, xliii, 1, 9.

<sup>18</sup> Laidlaw, P. P., and Dudley, H. W., *Brit. J. Exp. Path.*, 1925, vi, 197.

<sup>19</sup> Heidelberger, M., *Physiol. Rev.*, 1927, vii, 123.

# ON THE MECHANISM OF THE SERUM SENSITIZATION OF ACID-FAST BACTERIA.

BY STUART MUDD, M.D., AND EMILY B. H. MUDD.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

(Received for publication, April 15, 1927.)

The peculiar surface properties of acid-fast microorganisms make them readily susceptible of direct study by the interfacial tension method, a procedure not heretofore applied to analysis of the interactions of bacteria and sera. The acid-fast bacteria are thus especially favorable material for study of the mechanism of serum sensitization.

## *I. Directly Observable Alterations in the Bacterial Surface Produced by Sensitization.*

The surfaces of acid-fast bacteria grown on glycerol agar slants, and suspended in 0.8 per cent sodium chloride solution, as has already been pointed out, are usually vigorously wetted by a neutral oil, tricaprylin. When these bacteria are suspended in fresh sera and subsequently washed in salt solution and studied in a saline-tricaprylin interface, they are no longer easily wetted by the oil. They are on the contrary in equilibrium<sup>1</sup> in the oil-saline interface and pass into the oil only at the expense of mechanical work.

Strongly sensitized clumps of acid-fast bacteria may be carried ahead of the advancing oil phase, or, if the clump is adherent to the slide, it retards locally the advance of the oil and may even draw out the interface to form a "peninsula" or vacuole.<sup>2</sup>

---

<sup>1</sup> Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633, 647; 1926, xliii, 127.

<sup>2</sup> Similar phenomena have been described and figured for sensitized red blood cells (*J. Exp. Med.*, 1926, xliii, 127). However, the passage into the oil of clumps of sensitized acid-fast bacteria differs from that of sensitized erythrocytes in that no surface processes or "tails" are ever to be seen with the bacterial clumps.

Serum sensitization thus transforms the predominantly oil-miscible<sup>3</sup> surface of the acid-fast bacteria into a surface more readily wet by saline solution than by the oil. All stages of this transformation may be seen. A strong immune serum causes in low concentration just perceptibly decreased oil miscibility of its homologous microorganism. With increasing concentration of the sensitizing serum the resulting bacterial surface change usually increases to a maximum in the highest serum concentrations. Normal sera or immune sera with unrelated heterologous organisms cause detectable oil immiscibility in the higher concentrations only.

The *cohesion* of films of sensitized and unsensitized typhoid bacilli has been measured by Northrop and De Kruif; they found that, other factors being constant, the cohesion increased with the concentration of sensitizing immune serum, at least until maximum sensitization was reached.<sup>4</sup> This relation of sensitization to cohesion is very strikingly shown in the present experiments with acid-fast bacteria.

Suppose an even bacterial suspension virtually free of clumps to have been made, and equal portions mixed with serial dilutions of homologous immune serum. All tubes are centrifuged, the supernatant liquid decanted, and the sediment resuspended in salt solution. The sensitized bacteria resuspend in clumps whose size and coherence, as estimated from their resistance to dispersion by agitation, increase with the concentration of serum used for sensitization.<sup>5,6</sup> If now all tubes are centrifuged again and the sediment studied in the tricaprylin-saline interface the same relation of sensitization to cohesion is again brought out. The loose aggregates of unsensitized bacteria on contact with the advancing oil are explosively dispersed and flung into the oil. Weakly sensitized clumps are dispersed less completely, the dispersion occupies perceptible time, and the component bacteria are carried away from the clumps for greater distances along the the oil-saline interface before passing into the oil. Very strongly sensitized clumps may resist dispersion by the interfacial stresses altogether. In general, for any one bacterial suspension the degree of coherence of clumps produced by the sensitization and the degree of oil immiscibility produced by sensitization run roughly parallel.

The characteristic increase in resistance to wetting by oil produced by the serum sensitization of red blood cells and acid-fast bacteria has been called in an

---

<sup>3</sup> See Foot-note 11 in preceding paper.

<sup>4</sup> Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 664.

<sup>5</sup> Mudd, S., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 569.

<sup>6</sup> Mudd, S., *J. Immunol.*, 1927, xiii, 113.

earlier paper<sup>2</sup> "a positive stabilization reaction." A more convenient term is "positive interface reaction."

To summarize, serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is directly observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up the sediment (resuspension reaction) or by microscopic observation of the clumps in the saline-oil interface.

## II. Specificity of the Interface Reaction and Its Relation to Agglutinin Binding and Complement Fixation.

The procedure in obtaining the comparative titers given in this paper has been as follows:

The microorganisms were suspended in 0.8 per cent sodium chloride solution, with grinding when necessary, and the clumps thrown down by centrifugation. Portions of the relatively even supernatant suspension were mixed with serial dilutions of serum and put in the ice box overnight. The sera were unheated except where otherwise specified. The macroscopic *agglutination* readings were made in the morning. All tubes were centrifuged; the sediment was then either resuspended in the same serum dilutions, or else the serum was decanted and the sediment resuspended in a few drops of 0.8 per cent salt solution. The unsensitized bacteria resuspended evenly, the sensitized in clumps. This procedure has been termed the *resuspension* reaction. Its details and uses have been given in full elsewhere;<sup>6</sup> it is a simple and practical way of determining the binding of agglutinin by difficultly agglutinable strains. The bacteria were then washed once or more in salt solution and studied in the saline-tricaprylin *interface*. (Triolein was used as test oil in a few experiments.) Suspensions of the same strains and the same sera were used simultaneously by Dr. J. Furth in the *complement fixation* reaction.<sup>7,8</sup> The titer given is in each case unless otherwise indicated the highest dilution of serum which produced a perceptible effect.

---

<sup>7</sup> Furth, J., *J. Immunol.*, 1926, xii, 273.

<sup>8</sup> Mudd, S., and Furth, J., *J. Immunol.*, 1927, xiii, 369.



The binding of antibody as detected by the interface reaction has been found to be serologically specific and to indicate antigenic relationships among acid-fast bacteria which correspond to those indicated by agglutinin binding, and, except for the anomaly mentioned in the next paragraph, to those indicated by complement

TABLE I.

*Comparative Titers with a Saprophyte, an Avian and a Bovine Tubercle Bacillus, and Their Corresponding Antisera.*

		Antiserum against "milk" bacillus	Antiserum against Arloing bacillus	Antiserum against Bovine III
Suspension of "milk" bacillus	Agglutination	1:640++*	1:40+*	1:40+*
	Resuspension	1:640 flocculi*	1:2.5	1:5+ (others?)
	Interface reaction	1:80	1:1.25+, 1:10-	1:2.5
	Complement fixation	1:1000		
Suspension of Arloing bacillus	Agglutination	1:80	1:640++*	1:80
	Resuspension	1:5	1:640 flocculi*	1:160
	Interface reaction	1:2.5-	1:640	1:80
	Complement fixation		1:500	1:100
Suspension of Bovine III	Agglutination	1:80+*	1:40	1:320
	Resuspension		1:20	1:160
	Interface reaction	1:5	1:5- or ±	1:80
	Complement fixation	1:100	1:100	1:500

Moderate cross-reactions between *M. avium* (homogeneous culture of Arloing<sup>7</sup>) and *M. tuberculosis* (Bovine III strain). "Milk" bacillus practically unrelated serologically. All antisera from rabbits.

\* Titer not reached.

fixation. The specificity of the interface reaction is illustrated by Tables I and II.

The anomaly just referred to is that certain sera homologous with such difficultly agglutinable strains as virulent mammalian tubercle bacilli may give higher interface and agglutination titers with heterol-

ogous than with homologous microorganisms. Examples of such anomalous titers are given in Tables III and IV.

This anomaly has occurred when the heterologous was closely related to and much more easily agglutinable than the homologous strain. The complement fixation titers in such instances have been normal, *i.e.* higher with the homologous than with the heterologous strain. The crossed combinations, *i.e.* antiserum for easily agglutinable strain with homologous easily agglutinable and heterologous difficultly agglutinable strain, have likewise been normal for interface and agglu-

TABLE II.

*Comparative Titers with Three Species of "Tubercle" Bacilli of Cold Blooded Animals and Their Corresponding Antisera.*

		<i>M. marinum</i> antiserum	<i>M. chelonae</i> antiserum	<i>M. ranæ</i> antiserum
<i>M. marinum</i> suspension	Agglutination	1:80	All negative	1:20
	Resuspension	1:320	All negative	1:2½
	Interface reaction	1:160	1:5	1:10
	Complement fixation	1:1000	1:20 negative	1:20 negative
<i>M. chelonae</i> suspension	Agglutination	1:40	1:1280*	1:80
	Resuspension	All negative	1:320	1:10
	Interface reaction	1:2½±	1:80	1:5
	Complement fixation	1:50 negative	1:1000	1:100
<i>M. ranæ</i> suspension	Agglutination	1:5	1:10	1:2560*
	Resuspension	1:2½	1:10	1:2560*
	Interface reaction	1:2½	1:5	1:640
	Complement fixation	1:50 negative	1:50	1:2000*

Little or no serological relationship found between the three species. All antisera from rabbits.

\* Titer not reached.

tionation reaction as well as for complement fixation. Similar anomalies in agglutination experiments have been noted by Griffith<sup>9</sup> and by Wilson.<sup>10</sup> It may be necessary to use "absorption tests" in order to differentiate such strains with certainty.

The agglutination, resuspension, interface, and complement fixation titers have all been obtained for some forty-three combinations

<sup>9</sup> Griffith, A. S., *Tubercle*, 1924-25, vi, 417.

<sup>10</sup> Wilson, G. S., *J. Path. and Bact.*, 1925, xxviii, 1, 69.

TABLE III.

*Comparative Titers with Virulent Bovine<sup>8</sup> (S16) and Avirulent Bovine (III) Tubercle Bacilli, "Leprosy Bacillus" of Kedrowsky,<sup>7</sup> and Corresponding Antisera.*

		Bovine S16 antiserum	Bovine III antiserum	"Leprosy bacillus" of Kedrowsky antiserum
Bovine S16 suspension	Agglutination	1:5		
	Resuspension	1:80		
	Interface reaction	1:10		
	Complement fixation	1:1000		
Bovine III suspension	Agglutination	1:400	1:400	1:800
	Resuspension	1:100	1:200	1:400
	Interface reaction	1:200	1:100	1:200
	Complement fixation	1:500	1:500	1:100±
"Leprosy bacillus" of Kedrowsky suspension	Agglutination	1:200+*	1:200	1:800+++*
	Resuspension	1:25	1:50	1:800+*
	Interface reaction	1:12½	1:12½ negative	1:800
	Complement fixation			1:1000*

Higher interface and agglutination titers with easily agglutinable strains Bovine III and "leprosy bacillus" in Bovine S16 antiserum than with difficultly agglutinable Bovine S16 in Bovine S16 antiserum. Cross-reactions between Bovine III and "leprosy bacillus" show ordinary specificity. All antisera from rabbits.

\* Titer not reached.

TABLE IV.

*Comparative Titers with Avian (Egret) and Human<sup>8</sup> (H 37) Tubercle Bacilli and Corresponding Antisera.*

		Egret (avian) antiserum	H 37 antiserum
Egret (avian) suspension	Agglutination	1:400+++*	1:12.5+
	Resuspension	1:400 granular*	1:50
	Interface reaction	1:400+	1:12.5±
	Complement fixation	1:1000	1:20-50
H 37 human suspension	Agglutination	1:12.5±	1:12.5-
	Resuspension	Inconclusive	1:12.5
	Interface reaction	1:12.5-	1:12.5-
	Complement fixation	1:100	1:500

In H 37 antiserum agglutination and interface titers higher for egret strain than for H 37. Complement fixation shows ordinary specificity. Antisera from rabbits. Test oil triolein.

\* Titer not reached.

of serum and bacterial suspension. The interface reaction proves to be the least sensitive, *i.e.* to have the lowest average titer. The ratios of the average titers of the several reactions to that of the interface reaction are:

Agglutination titer:interface titer::3.5:1

Resuspension titer:interface titer::2.4:1

Complement fixation titer:interface titer::13.6:1

The resuspension titer:interface titer ratio has been less inconstant than the others. Moreover in those cases already cited (for instance Tables III and IV) in which the agglutination and resuspension titers were anomalous and the complement fixation titers normal, the interface titers paralleled the agglutinin binding and not the complement fixation titers. Since, therefore, agglutination as estimated by the improved or resuspension method, cohesion as estimated by the resuspension and interface methods, and the bacterial surface change (decreased oil miscibility) detected by the interface reaction all show general parallelism, it seems safe to conclude that the bacterial surface change, like the other effects, is at least in considerable part due to the binding of agglutinins by the bacterial surface.

Similar decrease in surface oil-miscibility and increase in cohesion has been found as a result of serum sensitization of mammalian red blood cells, but in this case the surface change was correlated with the binding of hemolytic sensitizer and was relatively independent of agglutinin binding.<sup>2</sup>

The importance of the physicochemical nature of the bacterial surface itself in agglutination is particularly well shown with the acid-fast bacteria. It has already been pointed out that agglutination as ordinarily detected may be entirely absent under circumstances in which the resuspension and interface reaction show abundant agglutinin to have been bound by the bacterial surface.<sup>6,11</sup> Conversely, with readily agglutinable strains agglutination may be complete in tubes in which interfacial observation shows that only a part of the bacterial surface has been altered by deposit of antibody. The latter point is illustrated in Tables V and VIII.

<sup>11</sup> It has repeatedly been shown also that inagglutinable bacteria may adsorb agglutinins (Buchanan, R. E., *J. Bact.*, 1919, iv, 82).

TABLE V.  
 "Milk" *Bacillus Suspension with Homologous Rabbit Antiserum.*

	Final serum concentration						Control
	5 per cent	2.5 per cent	1.2 per cent	0.62 per cent	0.31 per cent	0.16 per cent	
Agglutination.....	Complete	Complete	Complete	Complete to +++	+++	++	Tr.
Resuspension.....	All bacteria are in one or more great masses				Even suspension with some gross flocculi	Even suspension with some gross flocculi	Even suspension
Interface reaction.....	++++	++	+	-	-	-	-



TABLE VII.

*Titration of Human and Rabbit Sera against Human and Bovine Tubercle Bacilli, Virulent Strains P 15 B and Bovine C.*

		Final serum concentration									
		80 per cent	40 per cent	20 per cent	10 per cent	5 per cent	2.5 per cent	1.25 per cent	0.62 per cent	Control	
Human serum, Patient N. Br., with human bacteria	Agglutination	—	—	—	Slight tr.	Tr. to +	—	—	—	—	
	Resuspension	Medium flocculi + weak	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	—	—	—	—	—	—	—	—	—	
Human serum, Patient N. Bl., with human bacteria	Agglutination	—	—	—	—	Tr. to +	—	—	—	—	
	Resuspension	Fine flocculi +	Fine flocculi + weak	Even suspension + weak	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	—	—	—	—	—	—	—	—	—	
Normal rabbit serum I with human bacteria	Agglutination	Slight tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Slight tr.	—	
	Resuspension	Medium flocculi	Medium to fine flocculi	Very fine flocculi to even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	++	++ to +	+	+	+ weak	—	—	—	—	
Normal rabbit serum II with human bacteria	Agglutination	—	Tr.	Tr. to +	+	+	— to tr.	— to tr.	—	—	
	Resuspension	Medium to fine flocculi	Fine flocculi	Very fine flocculi	Some fine flocculi	Some fine flocculi	Some fine flocculi	Even suspension	Even suspension	Even suspension	
	Interface	++	+	+	+ weak	+ weak	+	—	—	—	
Human serum, Patient N. Br., with Bovine C bacteria	Agglutination	Tr.	Tr.	Tr.	Tr. to +	+	— to tr.	— to tr.	—	—	
	Resuspension	Very fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	—	—	—	—	—	—	—	—	—	

Human serum, Patient N. Bl., with Bovine C bacteria	Agglutination Resuspension	- Even sus- pension +	- Even sus- pension +	Tr. Even sus- pension -	Tr. to + Stringy bodies	Tr. Stringy bodies	Slight tr. Even sus- pension	- Even sus- pension
Normal rabbit serum I with Bovine C bacteria	Agglutination Resuspension	Tr. Fine floc- culi ++	+ Fine floc- culi	+ Fine floc- culi	Tr. to + Even sus- pension	- Even sus- pension	Tr. to + Even sus- pension	- Even sus- pension
	Interface		++ to +	+				
Normal rabbit serum II with Bovine C bacteria	Agglutination Resuspension	- Fine floc- culi +	Tr. to + Fine floc- culi + weak	Tr. to + Even sus- pension -	+ Even sus- pension	+ Even sus- pension	Tr. Even sus- pension	- Even sus- pension
	Interface							

Patient N. Br. pregnant and with four plus Wassermann at time blood was taken for tests tabulated. Chest negative.  
 Patient N. Bl. with negative Wassermann at time blood was taken and for at least a year previously. Chest negative.  
 Rabbits normal. Test organisms a virulent bovine tubercle bacillus, Bovine C, and a virulent human tubercle bacillus, P 15 B.



### *III. Effects of Sensitization with Normal Sera.*

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively indistinguishable by the methods used, at least, from the effects of immune sera. Sensitization with normal sera decreases the oil-miscibility and increases the cohesion of the acid-fast bacteria, but to a less degree and with lower titer than homologous immune sera.

Heating sheep, goat, rabbit, and human sera for 30 minutes at 56°C. usually decreased but did not abolish their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface. The test organisms used with normal sera have been *M. tuberculosis*: Strains H 37 (human), P 15 B (human), Bovine III, and Bovine C; "butter" bacillus; and *M. chelonci* (turtle). A titration is given in Table VI.

The sera of nine patients from the syphilis clinic have been tested against Bovine III.

These patients with the exception of two cases of arrested pulmonary disease were without clinical tuberculosis. The Wassermann reaction on the same serum specimen was four plus in two cases, was and had been negative in one case, and was negative or weakly positive but had formerly been four plus in the other six cases. The final serum concentration used for sensitization was in each case 80 per cent, and the test oil in the interface reaction was triolein. The sensitized bacteria, as in all other cases, were washed before study in the interface.

All these sera caused the characteristic bacterial surface change before, and all with one exception after heating for 30 minutes to 56°C. Another human serum, that of a girl of 12, negative for tuberculosis and with negative Wassermann, gave a two plus interface reaction after heating.

Titration of two human and two normal rabbit sera against a human and a bovine tubercle bacillus are given in Table VII. The test oil in this case was tricaprylin.

### *IV. The Agglutination Prezone.*

Since the important work of Northrop and De Kruif,<sup>12</sup> it has been recognized that the main features of bacterial agglutination can be

<sup>12</sup> Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 639, 655.

formulated in terms of the repellent forces of the electrical charges on the bacteria, which tend to keep them apart, and the cohesive force which tends to hold the bacteria together once they have collided. Substances which reduce the charge below a certain critical level ordinarily cause agglutination unless the cohesive force is also reduced.

The prezone in specific bacterial agglutination has not fitted well into this formulation. Studies by cataphoresis have indicated that the negative bacterial charge is reduced to a minimum in concentrated agglutinating sera.<sup>13</sup> Fig. 2 of Shibley's paper of 1924<sup>13</sup> is particularly instructive in this connection. This shows the bacterial surface charge falling with increasing serum concentration until the critical level is reached and agglutination occurs; further concentration of

TABLE VIII.  
*Egret Avian Suspension with Anti Egret Rabbit Serum.*

	Final serum concentration						NaCl
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent	
Agglutination	+	+	++	+++	++++	++++	-
Interface reaction	++++	++++	+++ or ++	+	+	+	-

Agglutination shows prezone, interface reaction does not.

serum reduces the charge along the same curve but agglutination ceases.

That the prezone is not due to lack of cohesiveness of the bacterial surface in concentrated sera is shown by our own results. Tables VII and IX of this paper and Tables IV and VI of an earlier communication<sup>6</sup> show that spinning the sensitized cells in the centrifuge abolishes the agglutination prezone. Similarly interfacial observation of the sensitized washed bacteria shows that the characteristic change in surface properties increases to a maximum after treatment

<sup>13</sup> Wells, H. G., *The chemical aspects of immunity*, New York, 1925, 137; Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 655; Shibley, G. S., *J. Exp. Med.*, 1924, xl, 457; Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124; Falk, I. S., and Jacobson, M. A., *J. Infect. Dis.*, 1926, xxxviii, 182; Shibley, G. S., *J. Exp. Med.*, 1926, xlii, 674.

TABLE IX.  
*Bovine III Suspension with Homologous Goat Antiserum.*

	Final serum concentration					
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent
Agglutination.....	+	+	++	+++	++++	++++
Resuspension.....	Very coarse floculi ++++	Very coarse floculi ++++	Floculi ++++	Floculi ++++	Floculi ++++	Even sus- pension +
Interface reaction....						Even sus- pension + weak

Agglutination shows prezone; resuspension and interface reactions do not.

with concentrated sera and is without prezone (Tables VII to IX of this paper, and Table IV of a previous paper<sup>6</sup>).

Since the agglutination prezone then is not due to a failure of concentrated sera to reduce the bacterial surface charge or to increase the cohesiveness of the bacteria and may be abolished by centrifugation, we suggest that the prezone in such cases is due to interference with the collisions of the bacteria which are of course prerequisite to their clumping. Whether the interference with the colliding of the bacteria is chiefly due to increased viscosity of the menstruum, or to an accumulation of excess colloidal material in the bacteria-serum interfaces is uncertain; possibly both effects play a part.

It seems that the observations cited in the preceding paragraph are incompatible with the conception of these prezones as due to combination of the bacteria with Ehrlich's "agglutinoids."<sup>14</sup>

Krumwiede, Cooper, and Provost<sup>15</sup> have reported the production of prezones or the exaggeration of existing prezones by addition of material containing products of bacterial autolysis or media constituents. These workers also attribute their prezones to interference with agglutination by excess colloidal material.

#### V. *Partial Reversibility of the Bacterial Surface Change.*

Since the pioneer observations of Hahn and Trommsdorff<sup>16</sup> and Landsteiner<sup>17</sup> a number of investigators have demonstrated the possibility of recovering antibodies by partial dissociation of antigen-antibody combinations.<sup>18</sup> In the acid-fast group Aronson<sup>19</sup> has reported partial recovery of complement-fixing antibodies from combination with *M. tuberculosis*, or with alcoholic or saline extracts of tubercle bacilli. It seemed probable therefore that subjecting the sensitized bacteria to conditions that have been described as favoring dissociation might cause alteration of the bacterial surface in the

<sup>14</sup> Park, W. H., and Williams, A. W., *Pathogenic microorganisms*, Philadelphia and New York, 8th edition, 1924, 192.

<sup>15</sup> Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 93.

<sup>16</sup> Hahn, M., and Trommsdorff, R., *Münch. med. Woch.*, 1900, xlvii, 413.

<sup>17</sup> Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905; Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

<sup>18</sup> Huntoon, F. M., *J. Immunol.*, 1921, vi, 117.

<sup>19</sup> Aronson, J. D., *Nat. Tuberc. Assn. Tr.*, 1922, xviii, 517.

direction of the normal. This expectation has been verified by experiment, and further evidence has thus been furnished that the changes observed to follow sensitization are consequent on combination of antibodies with materials in the bacterial surfaces.

Serial dilutions of serum were mixed with bacterial suspension and allowed to stand in the ice box overnight. The sensitized bacteria were then washed, resuspended in saline, and studied in the interface. Into certain tubes of the series 0.1 per cent  $\text{Na}_2\text{CO}_3$ ,<sup>19</sup> was distributed and into the other, usually alternate, tubes 0.8 per cent  $\text{NaCl}$ . Since we have not found evidence of appreciable dissociation by washing in  $\text{NaCl}$ , these saline tubes served as controls for the alkali-treated bacilli.<sup>20</sup> All tubes were returned to the ice box for five days or more. The tubes were then centrifuged, decanted, the sediment washed in  $\text{NaCl}$ , and studied in the interface.

Of ten series of sensitized bacteria prepared as described, six showed alteration of surface properties toward the normal as a result of treatment with  $\text{Na}_2\text{CO}_3$ . Results in the other four series were doubtful. Five of the positive instances are given in Table X.

Results of tests by resuspension were similar to those with the interface reaction. The sensitized bacteria digested with sodium carbonate and subsequently washed and resuspended in sodium chloride, were regularly in finer clumps than those in the alternate tubes digested with sodium chloride, as illustrated in Table XI. This result it is true is of less significance than the very convincing dissociation of antibody detected by the interface reaction, because the alkali might conceivably have dispersed the bacterial clumps simply through increasing the negative electric charge of the material on the bacterial surface. However, the only mechanism that, to our mind at least, satisfactorily explains both the observed interface and resuspension results after sodium carbonate treatment is dissociation of antibody from the bacterial surface.

#### *VI. What Chemical Groups Are Responsible for the Bacterial Surface Change?*

The evidence already cited indicates clearly, we believe, that fat-soluble material in the bacterial surface is coated over by the sensitiz-

<sup>20</sup> Indeed the impression has been gained that bacteria either normal or sensitized kept 24 hours or longer in  $\text{NaCl}$  tend to become slightly less oil-miscible.

ing serum antibodies or possibly by serum substances secondarily bound by these antibodies.<sup>21</sup> This shows that the actual chemical

TABLE X.

*Dissociation of Antibodies by Sodium Carbonate with Return of Bacterial Surface toward Normal.*

Tubes	Sensitized bacteria before treatment	Sensitized bacteria after treating with Na <sub>2</sub> CO <sub>3</sub>	Sensitized bacteria after treating with NaCl
A 1	++++	±	
A 2			+++
A 3	++	-	
A 4			+
B 1	++	+	
B 2	++ to +		++
C 1	++	+	
C 2	+	-	
D 1	++++	++	
D 2			+++
E 1	++	+	
E 2			++
E 3	++ to +	±	
E 4			+

+ to ++++ indicate degrees of positive interface reaction. - indicates a normal (unsensitized) bacterial surface.

Series A (Bovine C), *M. tuberculosis* sensitized with homologous rabbit anti-serum; serum concentration in Tube A 1, 20 per cent, in A 2, 10 per cent, etc.

Series B (Bovine III), *M. tuberculosis* sensitized with homologous goat anti-serum; serum concentration in Tube B 1, 10 per cent, in B 2, 5 per cent.

Series C, *M. ranæ* sensitized with normal horse serum; serum concentration in Tube C 1, 80 per cent, in Tube C 2, 40 per cent.

Series D, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit anti-serum; serum concentration in Tube D 1, 20 per cent, in D 2, 10 per cent.

Series E, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit anti-serum; serum concentration in Tube E 1, 5 per cent, in E 2, 2.5 per cent, etc.

<sup>21</sup> It is evident from the parallel complement fixation studies described in Section II above that complement is bound by the sensitized bacteria. To what extent this complement material contributes to the observed bacterial surface change is uncertain. Even from inactivated sera there is evidence to indicate

group<sup>22</sup> which combines with the antibody responsible for the interface reaction is at least very closely associated with fat-soluble material in the bacterial surface. It is not clear from these data, however, whether the binding group is a part of the lipin molecule, is conjugated with it, or is merely in very close physical association with the lipin.

Kurt Meyer<sup>23</sup> showed that alcohol extracts of the tubercle bacillus make active "antigens" for the complement fixation reaction. Wadsworth, Maltaner, and Maltaner<sup>24</sup> prepared extracts of tubercle bacilli with various fat solvents

TABLE XI.  
*Dissociation of Antibody by Treatment with Sodium Carbonate.  
Resuspension Reactions.*

	Final serum concentration			
	80 per cent	40 per cent	20 per cent	10 per cent
Sensitized bacteria before treatment	Medium flocculi	Medium flocculi	Medium flocculi	Medium flocculi
After treatment with Na <sub>2</sub> CO <sub>3</sub>	Almost even suspension		Even suspension with some medium flocculi	
After treatment with NaCl		Medium flocculi		Medium flocculi

*M. tuberculosis* (Bovine III) sensitized with serum of goat infected with Bovine C.

and found the acetone-insoluble lipoids to be potent complement fixation "antigens." Pinner has lately reported alcohol-soluble, acetone-insoluble sub-

that mid-piece (globulin) may be bound (Dean, H. R., *Proc. Roy. Soc. London, Series B*, 1911-12, lxxxiv, 416; Leschly, W., *Z. Immunitätsforsch., Orig.*, 1916, xxv, 219).

<sup>22</sup> We do not know how many antigens or antibodies are concerned in the sensitization process. The singular or plural nouns are used in this connection without implication.

<sup>23</sup> Meyer, K., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 359; xv, 245.

<sup>24</sup> Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1925, x, 241.

stances from tubercle bacilli to be active "antigens" in complement fixation and biologically active antigens when injected into rabbits.<sup>25</sup>

Freund, by agglutination and cataphoresis experiments, has demonstrated the possibility of sensitizing by serum the particles obtained by alcohol extraction of the tubercle bacillus.<sup>26</sup> This strongly suggests that a binding group is at least in some sort of chemical combination with the lipin.

Furth and Aronson<sup>27</sup> have shown 95 per cent ethyl alcohol extracts of a considerable number of acid-fast bacteria to contain serologically specific "antigens" for complement fixation. The writer has made some simple chemical tests with the extracts used by Furth and Aronson with the hope of gaining further light on the relation of the binding groups to the lipin.<sup>28</sup>

Amino acid tests with the material in these alcohol extracts were unsatisfactory. Millon's reaction was negative with suspensions of the residues from evaporation of the extracts. The possibility of traces of protein being present was not, however, satisfactorily excluded.

Molisch reactions with watery suspensions of the residues of these crude alcohol extracts were in all cases strongly positive. On hydrolysis positive orcinol-HCl tests for pentose were obtained.

The specific carbohydrate haptene of Mueller<sup>29</sup> and of Laidlaw and Dudley<sup>30</sup> likewise yields a pentose. The pentose in the alcohol extracts might conceivably be of nucleic acid origin. Nevertheless, finding such a carbohydrate and lipin in association in these serologically active and specific extracts suggests the possibility of harmonizing conflicting views in the literature on "lipoidal antigens." Possibly the "lipoidal antigens" of Meyer, Wadsworth, Maltaner, and Maltaner, Pinner, Freund, Dienes, and others and the surface lipid material involved in the interface reaction *may be conjugated lipins owing their serological specificity to a carbohydrate haptene*. Such an

<sup>25</sup> Pinner, M., *Am. Rev. Tuberc.*, 1925, xii, 142.

<sup>26</sup> Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124 (Tables 8 to 12).

<sup>27</sup> Furth, J., and Aronson, J. D., *J. Immunol.*, 1927, xiii, 265.

<sup>28</sup> Thanks are due these workers for the material and to Professor D. W. Wilson for aid in studying it.

<sup>29</sup> Mueller, J. H., *Proc. Soc. Exp. Biol and Med.*, 1924-25, xxii, 209; *J. Exp. Med.*, 1926, xliii, 9.

<sup>30</sup> Laidlaw, P. P., and Dudley, H. W., *Brit. J. Exp. Path.*, 1925, vi, 197.



hypothesis at least is free from the *a priori* objections to lipid antigens on the basis of their chemical simplicity.<sup>31</sup>

Dienes and his coworkers prepared an active alcohol extract of the tubercle bacillus. Partial separation from the lipoids increased the potency of the active part which they regarded as possibly identical with the carbohydrate haptene of Mueller and Laidlaw and Dudley.<sup>32</sup> Later this active non-lipoid group was interpreted as a haptene distinct from that of Mueller and Laidlaw and Dudley.<sup>33</sup>

It is of course well established that the tubercle bacillus contains protein antigenic material,<sup>34</sup> but with this we are not concerned in this study.

*The Sensitizing Material.*—It is common knowledge that the antibodies are intimately associated with the serum globulins and accompany the globulins through various chemical manipulations. Although considerable progress has recently been made by Felton<sup>35</sup> in defining the relations of antibodies to the globulin fractions, it remains uncertain still whether the antibodies are special sorts of globulins or are separate chemical substances attached to the globulins. However this may be, evidence has most recently been advanced by Shibley<sup>36</sup> to indicate that the binding of agglutinin by various bacteria gives them a surface coating of globulin. Shibley's figures indicate that globulin particles and bacteria which had fixed agglutinin from concentrated homologous antisera behaved identically in an electric field. That the globulin condensed on the bacterial surface is denatured is indicated both by analogy with the properties of other proteins condensed on surfaces and by the precipitation reactions of the sensitized bacteria themselves.

Through the kindness of Dr. L. D. Felton we have been able to compare the interfacial behavior of antipneumococcus globulins with the interfacial behavior of sensitized bacteria.

<sup>31</sup> Heidelberger, M., *Physiol. Rev.*, 1927, vii, 107.

<sup>32</sup> Dienes, L., and Schoenheit, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1925-26, xxiii, 106.

<sup>33</sup> Dienes, L., and Freund, J., *J. Immunol.*, 1926, xii, 137.

<sup>34</sup> Wells, H. G., De Witt, L. M., and Long, E. R., *Chemistry of tuberculosis*, Baltimore, 1923, 73.

<sup>35</sup> Felton, L. D., *J. Infect. Dis.*, 1925, xxxvii, 199, 309.

<sup>36</sup> Shibley, G. S., *J. Exp. Med.*, 1926, xli, 667.

A sample each of Type I, II, and III water-insoluble antipneumococcus horse serum protein which protects, agglutinates, and gives the rest of the immunological reactions,<sup>37</sup> was sent us by Dr. Felton. This material had been precipitated four times at the isoelectric point of the protein containing the protective substance and repeatedly washed with water.<sup>37</sup> As received the protein was suspended in distilled water in small aggregates of particles. The particles were about the size of bacteria and the aggregates in the dark field closely resembled small clumps of agglutinated microorganisms.

These globulins with immune properties were studied in the interface between distilled water and tricaprylin and between salt solution and tricaprylin. The saline suspension was prepared by cautiously adding 0.8 per cent sodium chloride solution to the protein in distilled water until the resulting mixture was only slightly cloudy. Much of the globulin was of course dissolved in so doing. The remaining aggregates did not scatter light so well as did the particles in distilled water; on contact with the interface they were quickly dispersed in the boundary plane; the resulting particles were visible for a moment spreading along the interface but were soon themselves dispersed until no longer visible. When a large aggregate was overtaken by a swiftly moving interface some of the protein dragged through into the oil with slight local retardation of the advancing interface.

In distilled water-tricaprylin interfaces, the globulin aggregates were in equilibrium. Large free floating aggregates were swept before the advancing oil with only that part of their surface which was contiguous to the boundary plane in the interface. Very small aggregates entered the interface and slid along it, but even with these the greater part of their mass was in the water rather than the oil. Small clumps adherent to the glass passed through the advancing interface, the latter being retarded locally; the aggregates were somewhat spread in passing through the boundary plane. Small clumps did not form peninsulas or vacuoles.

Globulin samples were denatured by bringing to a boil. The resulting soft coagula were broken up with glass rods and the flocculi studied in the distilled water-tricaprylin and saline-tricaprylin interfaces. The cohesion of the aggregates, their adhesion to the glass, and their resistance to passage into the oil were all plainly increased by denaturation. Even small aggregates were tightly stuck to the glass and passed through the interface without spreading but with formation of peninsulas and vacuoles.

Results with the globulins are summarized in Table XII.

It is to be noted that the changes in these globulins produced by heat denaturation were similar to those produced by heat injury of polymorphonuclear leucocytes and blood platelets.<sup>2</sup> This result is contrary to the expectation expressed in the earlier paper.

---

<sup>37</sup> Felton, L. D., personal communication.

The behavior of denatured globulin particles in the saline-tricaprylin interface and that of maximally sensitized acid-fast bacteria in such interfaces have been closely similar. Indeed we could not detect differences. This evidence is in harmony with the conclusion proposed by Shibley on the basis of cataphoretic and precipitation data that bacteria maximally sensitized with agglutinating serum are coated with denatured globulin. Much more extensive test of this important generalization is desirable.

TABLE XII.

*Interfacial Behavior of Globulins with Immune Properties.*

	Cohesion of globulin	Adhesion to glass	Resistance to passage from aqueous to oil phase	Behavior of aggregates in interface
Native globulin in NaCl solution	Slight	Slight	Slight	Aggregates are dispersed along interface
Native globulin in distilled water	Moderate	Moderate	Moderate	Aggregates at oil-water boundary largely in water; are somewhat spread in passing through interface
Denatured globulin in NaCl or distilled water	Great	Great	Great	Aggregates at oil-water boundary almost entirely in aqueous phase. No spreading of aggregates by interface

The interfacial behavior of the denatured globulins in saline solution was indistinguishable from that of maximally sensitized acid-fast bacteria in saline solution.

## SUMMARY.

Serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up

the sediment (resuspension reaction), or by observation of the clumps in the saline-oil interface.

The interface reaction is serologically specific and confirms the existence of qualitative differences among acid-fast bacteria.

The interface reaction parallels the binding of agglutinins as detected by the resuspension reaction, but not agglutination as ordinarily tested for. The interface reaction is less sensitive,—*i.e.*, gives lower titers—than the resuspension reaction in about the average ratio of 1:3. The interface reaction in most instances runs approximately parallel to the complement fixation reaction; under at least one set of conditions, however, the interface reaction is correlated with the binding of agglutinin but not with the complement fixation reaction. How much of the bacterial surface must be covered with agglutinin in order to produce agglutination varies greatly with the bacterial strain used.

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively not observably different from the effects of immune sera.

Heating normal human, sheep, goat, or rabbit sera for 30 minutes at 56°C. has usually diminished but not abolished their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface.

The agglutination prezone is shown to be due to interference by excess colloidal material with the collisions of the bacteria prerequisite to clumping. The prezone may be abolished by centrifugation and resuspension of the sediment.

Antibodies may be partially dissociated from the sensitized bacteria by alkali, with return of the bacterial surface toward its normal, unsensitized condition.

A carbohydrate yielding on hydrolysis a positive pentose test has been detected in the specific alcohol extracts of acid-fast bacteria studied by Furth and Aronson.<sup>27</sup> The tentative suggestion is made that the alcohol-soluble antigens of acid-fast microorganisms may be conjugated lipins owing their specificity to carbohydrate haptenes.

Protective antipneumococcus globulins after heat denaturation have shown behavior in the saline-tricaprylin interface indistinguishable from that of maximally sensitized acid-fast bacteria. This strengthens the evidence suggesting that sensitized bacteria are coated with denatured globulin.



## IMMUNIZATION EXPERIMENTS WITH LECITHIN.

By P. A. LEVENE, M.D., K. LANDSTEINER, M.D., AND J. VAN DER SCHEER.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, March 30, 1927.)

In the case of Forssman's heterogenetic antigen it was found possible to incite the production of antibodies by the injection of its specific component along with proteins. In a similar manner immune bodies were obtained with alcoholic extracts of blood corpuscles (1) and of organs (2). Since it is assumed generally though not proven that the specific part of the heterogenetic antigen is of lipoid nature it was natural to test the immunizing properties of lipoids of known chemical constitution. This was undertaken by Sachs and Klopstock (3) with lecithin and cholesterol. Indeed these authors state that they obtained antibodies by injecting into rabbits emulsions of lecithin, or cholesterol, containing pig serum. The tests were carried out mainly by means of complement fixation. A confirmatory paper was published by Ornstein (4) who also reported on successful immunizations with cephalin and with cerebroside.

There are some points in the observations of Sachs and Klopstock not easily understood on the basis of their assumptions. Their anti-lecithin serum reacted on lecithin Merck but it also reacted on cholesterol and even more intensely on the latter than on a certain lecithin preparation of higher purity than lecithin Merck. This phenomenon is ascribed by Sachs and Klopstock to the presence of some cholesterol in the injected antigen but against such a view may be pointed out that in the experiments of these authors it was rather difficult to produce sera which react upon cholesterol, by injections of this substance. Another difficulty arises from the fact that the purer of the two lecithin preparations employed was the less active. The explanations for these peculiarities offered by Sachs and Klopstock do not settle conclusively the questions at issue. Since the production of antibodies for well known lipoids would be of great

significance, it seemed desirable to us to repeat the experiments with various lecithins prepared by ourselves.

#### EXPERIMENTAL.

We injected three lots of five rabbits each, with ox brain lecithin, egg lecithin and hydro egg lecithin, respectively. In addition sera were prepared with a commercial egg lecithin preparation (Merck) as used by Sachs and Klopstock.

*Lecithin Preparations.*—Lecithin No. 1. Alcoholic extract of egg yolk was treated with a 25 per cent solution of cadmium chloride in methyl alcohol, the precipitate extracted twice with ether and decomposed with methyl alcohol containing 25 per cent of ammonia. The solution was concentrated and the residue was extracted with cold alcohol. The lecithin was again precipitated as a cadmium salt and the latter was repeatedly washed with ether. The cadmium salt was then treated with methyl alcohol containing ammonia gas; the solution was concentrated, taken up in a minimum quantity of ether and precipitated with acetone.

Analysis: C 64.6; H 10.49; N 2.17; P 3.96; amino N 0.

Lecithin No. 2. 15.0 gm. of lecithin No. 1 were dissolved in methyl alcohol, acetone was added until a sample on cooling to  $-5^{\circ}\text{C}.$  showed the formation of a precipitate. The entire solution was then brought to a temperature of  $-5^{\circ}\text{C}.$  and the precipitate formed was removed by centrifugalization. The mother liquor was concentrated nearly to dryness, the residue was taken up in ether and the solution was poured into an excess of acetone. The yield was 11.0 gm. This material was dissolved in 20.0 cc. of methyl alcohol, 20.0 cc. of water were added and the solution was adjusted to pH 4. Acetone was added so long as a precipitate formed. The yield was 9.0 gm.

Analysis: C 65.3; H 10.61; N 2.00; P 4.12.

Egg Lecithin No. 3. The cadmium salt, prepared as No. 1, was extracted with ether 8 times. The lecithin obtained in this manner was further purified as follows: 26.0 gm. of lecithin were dissolved in 40.0 cc. of ether, 40.0 cc. of 10 per cent acetic acid were added, the mixture was shaken for 1 hour and the lecithin precipitated with 500 cc. of acetone.

Analysis: C 66.00; H 10.59; N 2.03; P 3.90; amino N 0.

Brain Lecithin. The cadmium salt was decomposed as usual, the filtrate was concentrated, and taken up in ether; acetone was added until a small precipitate formed. This was removed by filtration. The filtrate was concentrated nearly to dryness, the residue was taken up in ether, acetone was added to the solution to incipient opalescence. The solution was chilled to approximately  $-8^{\circ}\text{C}.$  A precipitate formed which was removed by centrifugalization. The mother liquor was concentrated almost to dryness and taken up in a little ether. Acetone was added to opalescence and the solution brought to about  $-20^{\circ}\text{C}.$  A precipitate formed, which was separated by centrifugalization. It was then extracted with

acetone and dried under diminished pressure. It was preserved in an atmosphere of nitrogen gas.

**Hydrolecithin.** This was prepared from egg lecithin by reduction with hydrogen and colloidal palladium as a catalyst.

Analysis of Merck lecithin: C 65.41; H 10.55; N 1.81; P 3.41;  $\text{NH}_2\text{-N}$  0.

Tests were made also with a number of other samples of lecithin prepared in the laboratory with various modifications of the above described methods.

**Immunization.**—Rabbits were selected the sera of which gave no reactions in flocculation and complement fixation tests with emulsions of cholesterolized alcoholic beef heart extract<sup>1</sup> and of Merck's egg lecithin. 240 mg. of lecithin were emulsified with 15 cc. of saline and 3 cc. of pig serum diluted to 15 cc. with saline were added. This emulsion was kept at room temperature for 1 hour before injection.

The rabbits received intravenous injections of 5 cc. of the lecithin emulsion generally at intervals of 3 to 5, sometimes 7 days. The sera were tested several times during the course of the experiments, with both the lecithin used for injection and Merck's egg lecithin.

**Tests.** The complement fixation tests with the egg, brain and hydrolecithins were carried out as follows: To 0.25 cc. of progressively doubled dilutions of the inactivated serum starting with a dilution 1:5 were added 0.25 cc. of an emulsion of lecithin (prepared by fairly rapid addition of 24 cc. of saline to 1 cc. of a  $\frac{1}{2}$  per cent solution of lecithin in alcohol) and 0.25 cc. of guinea pig serum diluted 1:10. After incubation at 37°C. for 1 hour 0.25 cc. of sheep blood immune serum ( $2\frac{1}{2}$ –3 units) and 1 drop of a 50 per cent sheep blood suspension were added.

For the flocculation tests 1 part of a  $\frac{1}{2}$  per cent alcoholic solution of the egg lecithin was emulsified by fairly rapid addition of 5 parts of saline. 0.2 cc. of the emulsion was added to 0.2 cc. of the inactivated serum diluted 1:2 and the readings were taken after 20 hours standing at room temperature. For the flocculation tests with the brain lecithin and hydrolecithin a different procedure was adopted since emulsions prepared by the method described for egg lecithin were very unstable and were flocculated by most normal rabbit sera. 1 part of a  $\frac{1}{2}$  per cent alcoholic solution of brain lecithin was added rapidly to 5 parts of distilled water and 0.2 cc. of this liquid was mixed with 0.2 cc. of the inactivated serum diluted twice with a 2.7 per cent salt solution. In the case of the hydrolecithin 1 part of a  $\frac{1}{2}$  per cent solution of the substance was added by drops to 5 parts of boiling distilled water. The emulsion was filtered hot. For the test the serum was diluted with distilled water instead of saline.

The strength of the reactions in the tests is indicated as follows: Complement fixation tests—0 = no hemolysis, tr = trace, w = weak, d = distinct, str = strong, vstr = very strong, ac = almost complete, c = complete hemolysis. Flocculation tests—0 = no flocculation, ftr = faint trace, tr = trace;  $\pm$ , +, ++, +++, etc.

<sup>1</sup> For the technique see: *J. Exp. Med.*, 1927, xlv, 467.



By immunizing with egg lecithin Merck we obtained four strongly and two weakly active sera among six rabbits after six injections. [1]

The sera obtained with brain lecithin and hydrolecithin gave no distinct flocculation or complement fixation with these preparations

TABLE I.

Number of sera	Injections made with:	Flocculation with emulsions of:			
		Merck egg lecithin	Merck egg lecithin freed from cholesterol	Lecithin No. 1	Lecithin No. 1 with addition of 12 per cent cholesterol
966	Egg lecithin No. 1	0	0	0	tr
967	" " " 1	+±	0	0	+
968	" " " 1	0	0	0	tr
969	" " " 1	0	0	0	0
970	" " " 1	0	0	0	0
810	Merck's egg lecithin	+++	++±	0	±
Normal rabbit No. 1		0	0	0	tr
Normal rabbit No. 2		0	0	0	±

TABLE II.

Numbers of sera	Injections made with:	Complement fixation with an emulsion of egg lecithin No. 1	Complement fixation with an emulsion of Merck egg lecithin
966	Egg lecithin No. 1	c,c,c,c,c	c,c,c,c,c
967	" " " 1	c,c,c,c,c	0, tr, c, c, c
968	" " " 1	c,c,c,c,c	c,c,c,c,c
969	" " " 1	c,c,c,c,c	c,c,c,c,c
970	" " " 1	c,c,c,c,c	c,c,c,c,c
809	Merck's egg lecithin	c,c,c,c,c	0,0,0,0,ac,c
810	" " "	vstr,ac,c,c,c	0,0,0,0,0,ac,c
Normal rabbit		c,c,c,c,c	c,c,c,c,c

or with Merck's egg lecithin after twelve injections. Occasionally weak flocculations were noticed but after further injections the reactions disappeared.

The tests with the sera taken after twelve injections of egg lecithin No. 1 are presented in Tables I and II. Complement fixation tests

TABLE III.

Emulsions used for the tests:

A. 24 cc. of saline were added fairly rapidly to 1 cc. of a  $\frac{1}{2}$  per cent alcoholic solution of egg lecithin No. 1. This preparation gave no Liebermann reaction for cholesterol.

B. This emulsion was made as A, with 1 cc. of a  $\frac{1}{2}$  per cent alcoholic solution of egg lecithin No. 1 to which had been added 0.06 cc. of a 0.25 per cent alcoholic solution of cholesterol, corresponding to 3 per cent of the weight of lecithin.

C. Emulsion made as in A, with 1 cc. of egg lecithin No. 1 to which had been added 0.06 cc. of a 1 per cent alcoholic solution of cholesterol, corresponding to 12 per cent of the weight of lecithin.

D. As A, with Merck's egg lecithin. This preparation was found by the Liebermann test to contain 1.5 per cent of cholesterol or less.

E. As A, with Merck's egg lecithin from which the cholesterol was removed by dissolving 2 gm. in 15 cc. of ether and reprecipitation with 30 cc. of acetone. This purification was repeated twice. The product gave no Liebermann reaction.

F. 1 cc. of a  $\frac{1}{2}$  per cent alcoholic solution of cholesterol was slowly added to 24 cc. of boiling distilled water and the emulsion was filtered hot (method of Keiser (5)). The dilution of the sera was made with 1.8 per cent salt solution.

Number of sera	Injections made with:	Complement fixation with emulsions of:					
		Egg lecithin No. 1	Egg lecithin No. 1 + cholesterol	Egg lecithin No. 1 + cholesterol	Merck's egg lecithin	Merck's egg lecithin after removal of cholesterol	Cholesterol
967	Egg lecithin No. 1	A	B	C	D	E	F
809	Merck's egg lecithin	c, c, c, c, c	c, c, c, c, c	ac, c, c, c, c	0, 0, ac, c, c	0, 0, ac, c, c	0, 0, d, ac, c
	"	c, c, c, c, c	c, c, c, c, c	str, c, c, c, c	0, 0, 0, 0, str, c	0, 0, 0, w, c, c	0, 0, 0, 0, d, ac, c
810	Normal rabbit No. 1	vstr, ac, c, c, c	ac, c, c, c, c	d, vstr, c, c, c, c	0, 0, 0, 0, str, ac	0, 0, 0, 0, w, c, c	0, 0, 0, 0, str, ac
	Normal rabbit No. 2	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c
		c, c, c, c, c	ac, c, c, c, c	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c	c, c, c, c, c

performed after five injections were negative as well as flocculation tests at various other times.

The serum No. 967 which gave moderate reactions, as can be seen from Tables I and II, was tested against emulsions differing in their cholesterol content in comparison with sera prepared with Merck's egg lecithin (Table III).

The highly active sera resulting from the injection with lecithin Merck gave uniformly negative tests by the method of complement

TABLE IV.

Immune sera prepared by injections with Merck's egg lecithin	Complement fixations with emulsions prepared by addition of 24 parts of saline to a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	0,0,0,0,d,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 810	0,0,0,0,0,tr,c	vstr,c,c,c,c	ac,ac,c,c,c	ac,c,c,c,c
" 811	0,0,0,0,ac,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c
" 812	0,0,ac,c,c	c,c,c,c,c	c,c,c,c,c	c,c,c,c,c

Immune sera prepared by injections with Merck's egg lecithin	Flocculations of emulsions made by addition of 5 parts of saline to 1 part of a 1/2 per cent alcoholic solution of:			
	Merck's egg lecithin	Egg lecithin No. 1	Egg lecithin No. 2	Egg lecithin No. 3
No. 809	+++	±	0	0
" 810	+++	tr	ftr	ftr
" 811	++±	±	tr	0
" 812	+±	+	0	tr
Saline control	0	0	0	0

fixation with our egg lecithin preparations 1, 2 and 3. In the flocculation tests preparations 2 and 3 reacted faintly, No. 1 somewhat better, but considerably weaker than lecithin Merck (Table IV). The results with brain lecithin and hydrolecithin were similar. Some other of our preparations gave more distinct flocculation with the Merck lecithin immune sera but practically negative complement fixation as far as they were examined.

## SUMMARY.

In testing several egg lecithin preparations prepared by ourselves it was found that they did not react in complement fixation tests with immune sera made by injections with commercial egg lecithin Merck. With the flocculation method two of the preparations reacted only faintly. Also the brain lecithin and hydrolecithin gave no distinct reactions.

The immunization experiments of Sachs and Klopstock could easily be confirmed when commercial egg lecithin Merck was used for the injections. Immunizations with brain lecithin and hydrolecithin yielded no active sera. With an egg lecithin (No. 1) prepared by us the results were not satisfactory though a great number of injections was made. Only one serum gave reactions of medium strength by complement fixation and in flocculation tests with emulsions of Merck lecithin. It did not react however with the lecithin preparation No. 1 itself. In this respect the results resemble to a certain degree those of Sachs and Klopstock with their lecithin Böhringer immune serum. While the reactions of Merck lecithin were slightly diminished by the removal of cholesterol, addition of cholesterol to the lecithin No. 1 had no marked effect on the complement fixation tests, even when a larger amount was added than that present in the Merck preparation. The cholesterol content of this lecithin therefore does not suffice to account for the difference in the results. It is noteworthy that our lecithin immune serum No. 967 gave complement fixation with emulsions of cholesterol although this substance was not present in the injected material.

There are several plausible explanations for our results. According to one, the production of antibodies for lecithin would depend on certain physicochemical conditions of the emulsion injected or upon the presence of auxiliary substances in the lecithin preparation (*cf.* Sachs and Klopstock). Another possibility is that the active agent inducing the formation of antibodies is not lecithin itself but some other substance present in the active lecithin preparations. With regard to the latter assumption it may be mentioned that we obtained definite immunization effects from several injections of quantities

as little as 0.2 mg. of purified preparations of Forssman's heterogenetic haptene mixed with pig serum.<sup>2</sup>

To decide between the alternative explanations, further studies are necessary.

#### BIBLIOGRAPHY.

1. Landsteiner, K., and van der Scheer, J., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxii, 98; *J. Exp. Med.*, 1925, xlii, 123.
  2. Sachs, H., Klopstock, A., and Weil, A. J., *Deutsch. med. Woch.*, 1925, li, 589.
  3. Sachs, H., and Klopstock, A., *Biochem. Z.*, 1925, clix, 491.
  4. Ornstein, F., *Wien. klin. Woch.*, 1926, xxxix, 785.
  5. Keeser, E., *Biochem. Z.*, 1924, cliv, 321.
- 

<sup>2</sup> It may be stated that while in these experiments small quantities (1 mg.) of certain purified preparations of Forssman's heterogenetic haptene were active, no or a very slight effect was obtained on using larger amounts such as 100 mg. for each injection. These experiments will be fully described in a later communication.

# The Journal of General Physiology

Edited by

W. J. GROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

*The Journal of General Physiology* is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter.

*The Journal of General Physiology* is issued bimonthly, one volume of about 600 pages appearing in a year. Contributions should be sent to the editors of *The Journal of General Physiology*, The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y. The papers should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Papers will be printed as soon as possible after acceptance but not necessarily in the order in which they are received for publication. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (one volume) is \$5.00, payable in advance. Single copies cost \$1.00.

Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

## CONTENTS OF VOL. X, No. 5, MAY 20, 1927

- CLARK, HARRY. A theoretical consideration of the action of x-rays on the protozoan *Colpidium colpoda*.
- BRODY, SAMUEL. Time relations of growth. III. Growth constants during the self-accelerating phase of growth.
- MCCUTCHEON, MORTON, and LUCKE, BALDWIN. The kinetics of exosmosis of water from living cells.
- LUCKE, BALDWIN, and MCCUTCHEON, MORTON. The effect of salt concentration of the medium on the rate of osmosis of water through the membrane of living cells.
- MICHAELIS, L., ELLSWORTH, R. McL., and WEECH, A. A. Studies on the permeability of membranes. II. Determination of ionic transfer numbers in membranes from concentration chains.
- MICHAELIS, L., WEECH, A. A., and YAMATORI, A. Studies on the permeability of membranes. III. Electric transfer experiments with the dried collodion membrane.
- LILLIE, RALPH S. The activation of starfish eggs by acids. II. The action of substituted benzoic acids and of benzoic and salicylic acids as influenced by their salts.
- MURRAY, CECIL D. A relationship between circumference and weight in trees and its bearing on branching angles.
- REZNIKOFF, PAUL, and CHAMBERS, ROBERT. Micrurgical studies in cell physiology. III. The action of CO<sub>2</sub> and some salts of Na, Ca, and K on the protoplasm of *Amoeba dubia*.
- CHAMBERS, ROBERT, and POLLACK, HERBERT. Micrurgical studies in cell physiology. IV. Colorimetric determination of the nuclear and cytoplasmic pH in the starfish egg.
- WOLF, ERNET. Geotropism of *Agriolimax*.
- FENN, WALLACE O. The oxygen consumption of frog nerve during stimulation.
- HECHT, SELIG. The kinetics of dark adaptation.
- KUNITZ, M. Hydration of gelatin in solution.

Published by

The Rockefeller Institute for Medical Research

Avenue A and 66th Street, New York, N. Y.



# FURTHER OBSERVATIONS ON THE INABILITY TO TRANSMIT A RABBIT NEOPLASM BY CELL-FREE MATERIALS.

BY LOUISE PEARCE, M.D., AND JAMES B. MURPHY, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, April 21, 1927.)

The study of a malignant neoplasm of the rabbit which has been in progress in these laboratories for several years, included experiments in 1921 and 1922 in which it was found that propagation could not be accomplished by the use of filtrates of the tumor or of desiccated tumor tissue (1).

As serial transplantation progressed, adaptation to passage has been associated with an increase in the energy of cell growth as shown by alterations in the character of growth of the primary tumor and metastases which have resulted in a more rapid progress of the disease toward death or recovery (2). Under these circumstances, a repetition of the filtration and desiccation experiments was desirable for the purpose of checking the earlier results with material of a more favorable character as far as capacity for growth is concerned. A few experiments were also carried out with fluid media which had been in contact with tumor tissue and subsequently filtered or centrifuged in order to ascertain whether the tumor could be propagated by a cell-free agent obtained by diffusion. The present paper contains the results of these several experiments in the order named.

## EXPERIMENTAL.

### *Materials and Method.*

Tumors of the 56th, 65th and 68th generations were used in this study which was carried out in April and October, 1926, and in January, 1927. The material employed was obtained from primary testicular tumors, the growth activity of which was controlled by



intratesticular inoculations of fresh cell emulsions not subjected to any manipulation other than emulsification with normal saline; sand was not used. This procedure and route of inoculation are those used in the majority of experiments with this tumor. In 2 preliminary experiments with Hartley's broth, as described below, a control inoculation of the fresh tumor was not made; the condition of the tissue was such, however, that its transplantation as ordinarily carried out would undoubtedly have resulted in tumor growth.

*Filtration.*—Immediately after removal of the tumor, portions of it were pulped, pressed through a fine meshed sieve and ground with sand. Ringer's solution was added in the proportion of 50 cc. to approximately 5 gm. of tumor. The mixture was shaken for 20 minutes, centrifuged for 10 minutes at a speed of 1500 revolutions per minute and the supernatant fluid filtered through Berkefeld V candles. The cell-free state of the filtrate was controlled by the addition of a suspension of *B. prodigiosus* to the fluid prior to filtration.

A thin suspension of Kieselguhr was thoroughly mixed with the filtrate in the proportion of 0.5 or 1.0 cc. to 25 cc. of the filtrate and 1.0 cc. of the mixture was injected intratesticularly in normal rabbits.

*Desiccation.*—Small pieces of the tumor were pressed through a fine sieve and a thin layer of the pulp was spread on the bottom of large Petri dishes. The dishes were placed over concentrated sulfuric acid in desiccator jars and the air evacuated by a Geryk pump to a pressure of 3 mm. The jars were then kept in a freezing box at a temperature of  $-1^{\circ}\text{C}$ . for 5 days. The material was pulverized and taken up in a small amount of normal saline or of Ringer's solution; 1.0 cc. of this suspension was injected intratesticularly in normal rabbits.

*Supernatant Fluid of Tumor "Cultures."*—Cubes of tumor tissue measuring approximately 0.5 cm. along each side were placed in test-tubes containing 5 cc. of Hartley's KCl glucose broth and 1 cc. of fresh rabbit serum. The tubes were put in jars from which the air was evacuated, and the jars were kept in the ice box for 48 hours. At the end of this time, the supernatant fluid was centrifuged for 10 minutes at a speed of 1500 revolutions per minute, and filtered through a Berkefeld V candle, *B. prodigiosus* having been added before filtration. Inoculations of the filtrate were made into 1 or both testicles of normal rabbits, 0.5 cc. or 1.0 cc. being used, and in one experiment 0.2 cc. was also injected intracutaneously on the ventral surface of the sheath.

In one experiment, the supernatant fluid of the cultures was centrifuged twice but not filtered. Both intratesticular and intracutaneous injections were carried out with this material.

*Tissue of "Cultures."*—Tumor tissue which had been kept in Hartley's broth in the ice box for 48 hours was emulsified with normal saline and 0.4 cc. of the emulsion was injected into the testicles of normal rabbits.

*"Stored" Tissue.*—Pieces of the same tumor used for the above culture experiment were placed in small Petri dishes with bits of gauze soaked in normal saline, care being taken that the tissue did not come in contact with the gauze. The dishes were sealed with adhesive tape and placed in the ice box for 48 hours. Each piece of tumor was then emulsified with normal saline and 0.4 cc. of each emulsion was injected into both testicles of 5 normal rabbits.

TABLE I.  
*Results of Filtration Experiments.*

Experiment	Generation of tumor	Tumor filtrate						Controls—fresh tumor					
		No. of rabbits	Inoculation			Growth		No. of rabbits	Inoculation			Growth	
			Route	Number	Amount	Positive	Negative		Route	Number	Amount	Positive	Negative
I	65	10	R. testicle	10	1.0	0	10	10	R. testicle	10	0.3	10	0
			L. “	5	1.0	0	5						
II	68	5	R. “	5	1.0	0	5	5	“ “	5	0.3	5	0
			L. “	5	1.0	0	5						

TABLE II.  
*Results of Desiccation Experiments.*

Experiment	Generation of tumor	Desiccated tumor							Controls—fresh tumor							
		No. of rabbits	Duration of desiccation  days	Inoculation			Growth		No. of rabbits	Inoculation			Growth			
				Route	Number	Amount	Positive	Negative		Route	Number	Amount	Positive	Negative		
I	65	5	5	R. testicle	5	1.0	0	5	10	R. testicle	10	0.3	10	0		
II	68	5	5	"	"	5	1.0	0	5	5	"	"	5	0.3	5	0
				L.	"	5	1.0	0	5							

The rabbits were examined frequently in order to determine any reaction at the site of inoculation which could be diagnosed as tumor growth by inspection or palpation. The period of observation varied from 5 weeks to 3 months. With the intratesticular route of inoculation, the usual incubation period of this tumor at present is 5 to 8 days, at the end of which time there is no doubt of the active character of the growth.

In the experiments in which tumor filtrates or desiccates were employed, the rabbits were observed for 2 and 3 months, since it was probable that growth, if any occurred, would be greatly delayed. In the experiment in which centrifuged

TABLE III.  
*Results of Diffusion Experiments.*  
*Anaerobic Cultures in Hartley's Broth 48 Hours—Ice Box Temperature.*

Experiment	Generation of tumor	Supernatant fluid	Inoculation					Controls—fresh tumor				
			No. of rabbits	Site Number	Amount	Growth		No. of rabbits	Site Number	Amount	Growth	
						Positive	Negative				Positive	Negative
I	56	Centrifuged	5	Testicle 5	cc. 0.5 in 3 1.0 " 2	0	5			cc.		
				Sheath 5	0.2	0	5					
II	56	" and filtered	5	Testicle 5	0.5 in 3 1.0 " 2	0	5					
				Sheath 5	0.2	0	5					
III	68	Centrifuged and filtered	5	Testicle 10	1.0	0	10	5	Testicle 5	0.3	5	0

TABLE IV.  
*Results of Experiments with Stored Tissue.*

Procedure		Inoculation				Growth	
		No. of rabbits	Site	Number	Amount	Positive	Negative
Hartley's broth (anaerobic)	ice box 48 hrs.	5	Testicle	10	cc. 0.4	0	10
Stored (moist condition)	" " 48 "	5	"	10	0.4	9	1?

supernatant fluid of the tumor cultures in Hartley's broth was used, and in one of the experiments in which this fluid was filtered, the observation period was 34 days; in the remainder it was 2 months. In a few instances; the testicle or the skin

of the inoculation area was removed during the experiment in order to obtain additional evidence of the presence or absence of tumor growth from gross inspection or from the histological picture.

#### RESULTS.

These experiments which are summarized in Tables I to IV turned out entirely negative. No growth was obtained from any of the 25 injections (15 rabbits) of filtrates of tumor emulsions nor from the 15 inoculations (10 rabbits) of desiccated tumor. In like manner, no growth resulted from the inoculation of filtered or centrifuged Hartley's broth which had been in contact with tumor tissue in the ice box for 48 hours. There were 20 testicular and 10 intracutaneous injections carried out with these materials (15 rabbits). Furthermore, the tumor tissue which had been "cultured" in Hartley's broth failed to grow when injected into the testicles of 5 rabbits. On the other hand, the same tumor used in a cultivation experiment was still capable of active growth after being kept in the ice box for 48 hours under moist conditions. Primary tumors developed from 9 of the 10 inoculations made with this material.

These results therefore contrast sharply with those obtained in the control series of rabbits in which the material used for inoculation was not subjected to any manipulation other than emulsification. Primary growths were obtained in every instance from the inoculation of the same tumors which had been used for filtration, desiccation or cultivation in Hartley's broth.

#### DISCUSSION.

The results of the present experiments confirm the earlier observations in that it has not been possible to propagate this malignant neoplasm of the rabbit with Berkefeld filtrates of the tumor or with desiccated tumor tissue. Furthermore, no success attended the attempts to demonstrate an agent capable of growth which could be separated from the tumor cells by diffusion into a fluid nutrient medium as is the case with a filtrable chicken tumor.\*

It would appear therefore that as far as this neoplasm is concerned,

\* Unpublished experiments of Jas. B. Murphy.

it is reasonably certain that living cells are essential for its propagation. This deduction is supported by the results of the experiment in which active growths were obtained with tissue which had been kept in a moist condition in the ice box for 48 hours as contrasted with the failure to obtain growth from the inoculation of the same tissue which had been kept in Hartley's broth in the ice box for the same time. The probable explanation of this failure is the rapid autolysis of cells which occurs under the latter conditions.

It is significant in this connection to recall that the cells of this tumor resist supposedly deleterious influences to a remarkable degree. Repeated freezing and thawing, for instance, destroys most cells, judging from dark-field examination, but a few apparently intact cells may be recognized and intratesticular inoculation of tumor tissue subjected to these procedures is followed by tumor growth (1).

The first filtration and desiccation experiments were carried out with the 4th, 10th and 12th generations of tumor transplants, while growths of the 56th, 65th and 68th generations were used in the work now reported. Whatever changes have occurred in the growth capacity of the tumor cells incident to long continued transplantation there has evidently been no alteration in a hypothetical cell-free agent by which this agent would be more readily demonstrable with the procedures employed.

It is evident from the present experiments as well as from those previously reported, that there is an essential biological difference between this neoplasm of the rabbit and certain tumors of fowls which can be propagated with tissue filtrates or desiccates. This difference may possibly be a matter of animal species since the satisfactory demonstration of the filtrability of a mammalian tumor has yet to be made. It is not unlikely that the biological differences between such species as birds and mammals may extend to the occurrence of tumor agents distinct from tumor cells. On the other hand, the significant factor may be the type of cell involved since the fowl tumors are classified as sarcomata, while the rabbit neoplasm is considered to be of epithelial origin.

## CONCLUSIONS.

It has not been possible to propagate a malignant neoplasm of the rabbit with cell-free filtrates, or desiccated tumor tissue or by the use of fluid media kept in contact with tumor tissue. These findings confirm the results of previous experiments carried out with early generations of the tumor.

The existence of an agent distinct from the tumor cell which could initiate growth has not been demonstrated.

The experiments bring out an essential biological difference between this mammalian neoplasm which is considered to be of epithelial origin and certain filtrable tumors of fowls which have been classified as sarcomata.

## BIBLIOGRAPHY.

1. Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, **xxxvii**, 811.
2. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, **xl**, 583.  
Pearce, L., Brown, W. H., and Van Allen, C. M., *J. Exp. Med.*, 1924, **xl**, 603.



# ON A SPECIFIC SUBSTANCE OF THE CHOLERA VIBRIO.\*

BY K. LANDSTEINER, M.D., AND PHILIP LEVINE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 25, 1927.)

Rather extensive work has been devoted to the question of lipoid antigens in bacilli of the acid-fast group but there are also some reports concerning the solubility in organic solvents of antigens of other microorganisms. Among these are contributions dealing with *V. cholerae*.

Levaditi and Mutermilch (1) were able to prepare solutions of cholera antigen by mixing one volume of an extract in isotonic salt solution with five volumes of absolute alcohol. For the experiments the fluid was centrifuged and the supernatant was evaporated. The residue was found to contain active antigen when tested with anticholera serum in complement fixation tests. After 2 to 3 injections of 40 mg. each of the substance into rabbits the sera of the animals contained specific agglutinins, bactericidal substances, opsonins, and complement-binding antibodies. Guinea pigs treated with the substance acquired active immunity. The substance was insoluble in ether, acetone, or hot absolute alcohol. It resisted boiling in a water solution, and was not easily destroyed by dilute acid or alkali.

Prausnitz (2) in attempting to verify the findings of Levaditi and Mutermilch ascribed the effects observed by these authors to their method of filtration through paper which would not suffice to prevent the passage of bacilli into the filtrate. As a matter of fact after filtration through candles, the author was unable to demonstrate the presence of antigens in the extracts either by immunization or by tests *in vitro*.

In view of the divergent results recorded we undertook a renewed study of the subject.

## EXPERIMENTAL.

In order to determine the most suitable method for extraction cholera vibrios were treated successively with ether at room temperature for 24 hours and with various concentrations of alcohol by boiling under reflux for 1 hour. 10 cc. of solvent

---

\* See the preliminary report in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 248.



was employed for the 24 hour growth of each Blake bottle. The alcoholic solutions were filtered by means of a hot water funnel, evaporated on the steam bath to a small volume, the residue taken up in saline, and brought to a volume of 5 cc. per bottle. The solutions were put through common filter paper or kieselguhr paper (Macherey) and precipitin tests were made with the filtrates and cholera immune serum. There was no reaction with the extracts made with ether or strong alcohol but the extracts made with dilute (75 per cent) alcohol were found to be active.

Accordingly for the further work the following technic was adopted. The harvest of a 24 hour growth of cholera vibrio of 150 one quart Blake bottles was washed off with 1.5 liters saline. The microbes were centrifuged, washed twice with about 1-1.5 liters of saline and once with the same quantity of 50 per cent alcohol, centrifuging each time for 30 minutes at high speed.

The sediment was put into 95 per cent alcohol. After 1 or several days the centrifuged bacterial mass was boiled under reflux for 1 hour in 1.5 liters of absolute alcohol and filtered hot. The vibrios were then treated with boiling 75 per cent alcohol. At first two such extractions were made and the solutions joined. Subsequently the first and second extracts were kept separately. The first extraction was made with 500 cc. for 30 minutes; the second with 1500 cc. for 1 to 2 hours. The extracts were filtered through a hot water funnel. Because of the slow rate of filtration the filter paper had to be renewed several times. The hot filtrate ran through clear, but became turbid on cooling and when kept in the ice chest, a flocculent precipitate settled out.<sup>1</sup> This was separated by spinning in a cooled centrifuge and washed with some absolute alcohol and dry ether. From 150 Blake bottles the yield of the first and second extracts approximated 60 mg. and 300 mg. respectively.

It seems, according to preliminary experiments, that a better yield can be obtained by isolating the substance from water extracts.

In the manner described a substance was obtained in the form of a white to grayish white powder. In water the substance swells, and slowly a viscous, more or less turbid fluid is formed; it is more readily soluble on addition of a trace of alkali. The substance was precipitated by cholera immune sera up to dilutions of 1:500,000. In higher concentrations heavy flakes were formed. Both the substances from the first and second extractions in a 1 per cent solution gave positive biuret, xanthoprotein, and Millon reactions and precipitation with trichloroacetic acid, tannic acid, phosphotungstic acid, and sulfo-

<sup>1</sup> With another strain of *V. cholerae* and some other vibrios, the alcoholic extracts became faintly turbid on standing in the ice chest and flocculation occurred only after addition of a small quantity of salt solution.

salicylic acid. The reactions were considerably weaker with the second extract. Both preparations gave a strong Molisch test. On heating a 2 per cent solution in  $N/2$  HCl for 90 minutes on the steam bath the liquid became turbid and a rather voluminous precipitate separated. The yield of this precipitate was about 175 mg. per gm. of the hydrolyzed substance. The supernatant fluid gave strong reduction with Fehling's solution and with phenylhydrazine an osazone crystallizing in needles, no pentose reaction with orcinol.

After 10 hours heating the content in reducing sugar was found to be 20.5 per cent, calculated as glucose. After oxidation with nitric acid the solution gave an intense reaction for phosphoric acid.

The precipitate appearing on hydrolysis showed acid character in that it was soluble in alkali, and could be precipitated from the solution by acid. It could be separated by means of methyl alcohol in two parts, one soluble in methyl alcohol and in ether, and another insoluble in these solvents. The former gave the values (calculated for ash-free substance): C, 67.03; H, 10.28; N, 1.35: the latter C, 55.83; H, 8.52; N not determined.

For testing the antigenic properties, the dry substance obtained by alcoholic extraction of the vibrios was dissolved in saline and injected into rabbits. After 2 to 3 injections of 2.0 mg., and also 0.2 mg., precipitins were formed for the extracted substance as well as agglutinins for *V. cholerae*. The antigenic activity of still smaller quantities was not tested.

The substance proved to be toxic and there was loss of animals in the immunization experiments. Guinea pigs died after intravenous injection of 1.0 mg. of the substance and sometimes even 0.1 to 0.2 mg. was lethal.

In order to exclude the presence of bacilli in the injected material immunization experiments were also carried out with 75 per cent alcoholic extracts filtered hot through Berkefeld candles tested for impermeability to a broth culture of *V. cholerae*. The precipitate settling after cooling was employed in quantities of 2 mg. per injection. The results were essentially identical with those recorded in Table III.

The properties of our material bring to mind the specific bacterial substances studied by Avery and Heidelberger on account of the content in carbohydrates, but it differs by virtue of its antigenic

activity and the presence of protein. Consequently efforts were made to determine whether a specifically reacting non-antigenic substance—a haptene according to our nomenclature—could be separated. In this we succeeded in the following manner.

The first alcoholic extract, richer in protein, was discarded. 1 gm. of dry substance obtained in the second extraction was taken up in 10 cc. of water and

TABLE I.  
*Precipitation Tests.*

Antigens: A = crude extract; B = purified product obtained from A in the manner described.

To 0.2 cc. of the antigen dilutions was added 1 capillary drop of immune serum; readings after 5 minutes and 1 hour. The immune serum was obtained by injections of cholera vibrios into a rabbit.

The agglutinin titer of this serum was 1:8000. The intensity of the reactions is indicated as follows: f. tr. = faint trace; tr. = trace;  $\pm$ , +,  $+\pm$ , etc.

	Antigen	Antigen diluted 1:					Readings after
		5000	50,000	250,000	500,000	1,000,000	
Cholera immune serum No. 82	A	$++\pm$	+	tr.	0		5 min.
		$++++$	$+\pm$	$\pm$	f. tr.	0	1 hr.
	B	$+++$	+	tr.	0		5 min.
		$++++$	$++$	$\pm$	tr.	0	1 hr.
Normal rabbit serum	A	0	0				1 hr.
	B	0	0				1 hr.

In 24 hour readings the precipitate seemed to be partly dissolved especially in the tubes with higher concentrations of antigen.

to it was added 40 cc. of N/10 NaOH. After about 1 hour, the solution was centrifuged from some insoluble material, and 3 volumes of alcohol added. In the supernatant fluid which was kept for several hours, a second precipitate formed on addition of 1 more volume of alcohol. The first precipitate was dissolved in 25 cc. of water, neutralized, and a little  $\text{Na}_2\text{CO}_3$  added whereupon a slight precipitate formed. After centrifuging the supernatant fluid was acidified (weakly acid to Congo red) with acetic acid so that on addition of 2 volumes of alcohol a flocculating precipitate appeared. The precipitate was washed with 95 per cent alcohol and dried with absolute alcohol and ether. The yield was about 0.4 gm.

TABLE II.

## Precipitation Tests.

The antigens used are the crude bacterial extracts prepared in the manner described. Immune serum 32 was obtained by 3 injections each of 2.0 mg. of the crude cholera extract into a rabbit.

Extract prepared from	<i>V. cholera</i> immune serum 82						<i>V. cholera</i> extract immune serum 32						<i>B. paratyphosis</i> B immune serum 74						Normal rabbit serum							
	Antigen diluted 1:																									
	1000	5000	50,000	250,000	500,000	f. tr.	1000	5000	50,000	250,000	500,000	1000	5000	50,000	250,000	500,000	1000	5000								
<i>V. cholera</i> (Krumwiede)	+++	+++	+++	+++	+++	f. tr.	+++	+++	+++	++	+	+	+	+	+	+	+	+	0	0						
<i>V. cholera</i> (Wadsworth)	+++	+++	+++	+++	+++	f. tr.	+++	+++	+++	++	+	+	+	+	+	+	+	+	0	0						
<i>V. metchnikovi</i>	f. tr. ?	0	0	0	0	0	+++	+++	++	+	+	+	+	+	+	+	+	+	0	0						
<i>V. tyroginus</i>	f. tr. ?	0	0	0	0	0	f. tr. ?	0	0	0	0	0	0	0	0	0	0	0	0	0						
<i>V. Finkler-Prior</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
<i>B. paratyphosis</i> B	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	f. tr.	0						

This product was a white powder, slowly swelling and dissolving in water, yielding a faintly turbid liquid in a 1 per cent solution. On addition of a trace of alkali it dissolved more readily. The solution

TABLE III, *a*.  
*Precipitation Tests.*

Rabbits injected with A = crude extract: B = purified product obtained from A in the manner described.

Injected with	Rabbit No.	Substance A diluted 1:								Read after
		500	5000	50,000	125,000	250,000	500,000	1,000,000	2,000,000	
A	32	—	++++	+	±	tr.	0			1 hr.
		—	++++	++++	±±	±	tr.	0		24 hrs.
	33	—	+++	+	tr.	0				1 hr.
			++++	++++	±±	±	f. tr.	0		24 hrs.
	41	—	++++	+	±	f. tr.	0			1 hr.
			++++	++	+	±	f. tr.	0		24 hrs.
B	90	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	91	0	0	0						1 hr.
		tr.	0	0						24 hrs.
	92	0	0	0						1 hr.
		f. tr.	0	0						24 hrs.
<i>V. cholerae</i>	82	—	++++	±±	±	±	f. tr.	0		1 hr.
		—	++++	++++	++	+	±	tr.	0	24 hrs.
Normal rabbit serum	1	0	0	0						1 hr.
		0	0	0						24 hrs.
	2	0	0	0						1 hr.
		0	0	0						24 hrs.

gave a negative biuret and Millon's reaction, a very slight xanthoprotein reaction, faint traces of turbidity with trichloroacetic, tannic acid, and sulfosalicylic acid, and no turbidity with phosphotungstic acid.

With Molisch's reagent it reacted intensely. A 2.5 per cent solution in N/50 sodium hydroxide gave a rotation of  $+16$  in a 5 cm. tube. An

TABLE III, *b*.  
*Agglutination Tests.*

To 0.5 cc. of the stated dilutions of inactivated serum was added 0.5 cc. saline suspension of heat-killed *V. cholerae* grown for 18 hours on agar slants. The tubes were kept for 2 hours at  $37^{\circ}$  and overnight in the ice box. A = crude extract; B = purified product obtained from A in the manner described.

Injected with	Rabbit No.	Serum diluted 1:							Read after
		50	500	1000	2000	4000	8000	16,000	
A	32	—	++++	+++	++±	+	0		2 hrs.
			++++	++++	+++	±	±	0	24 hrs.
	33	—	+++	+++	+	f. tr.	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
	34	—	++++	+++	±	±	0		2 hrs.
			++++	++++	+++	+	tr.	0	24 hrs.
B	90	0	0	0					2 hrs.
		0	0	0					24 hrs.
	91	0	0	0					2 hrs.
		0	0	0					24 hrs.
	92	0	0	0					2 hrs.
		0	0	0					24 hrs.
<i>V. cholerae</i>			++++	++++	+++	±	tr.	0	2 hrs.
			++++	++++	++++	+	+	0	24 hrs.
Normal rabbit serum	1	0	0	0					2 hrs.
		0	0	0					24 hrs.
	2	0	0	0					2 hrs.
		0	0	0					24 hrs.

analysis gave the following values calculated for ash-free material: C, 49.05; H, 7.17; N, 4.34; P, 1.67; no S; ash, 4.98. The second precipitate mentioned above, after reprecipitation with alcohol in acid

solution, analyzed as follows: C, 47.04; H, 7.09; N, 4.31; P, 1.66; ash, 2.84.

When hydrolyzed with  $N/2$  HCl, sugar could be demonstrated by Fehling's solution and by the osazone test, and a precipitate appeared as in the experiments mentioned above.

With cholera immune serum it was precipitated to the same titer as the original product but rather more intensely (Table I). The precipitin reaction was not diminished by heating a 2 per cent solution for 1 hour in the steam bath; neither was it appreciably affected by digestion with pepsin or trypsin. It resisted the action of nitrous acid.

The specificity of the substance was investigated with the results shown in Table II.

The tests demonstrate the specificity of the reaction with the precipitable substance. A third strain of cholera gave a product which reacted only weakly with cholera immune serum. This strain was atypical in that it showed spontaneous agglutination and was considerably less agglutinated by immune serum than the other strains.

The antigenic activity of the crude and purified extract was tested as follows:

Two batches of rabbits were injected—one with the crude material used for the purification; another with the purified substance. Three injections each of 2 mg. of the substances dissolved in 1 cc. saline were given intravenously at intervals of 5 days. 7 days after the last injection the sera were tested. The results are presented in Tables III, *a* and III, *b*. Before the injections the sera of the animals did not agglutinate in dilutions of 1:10 and 1:50 and gave no precipitin reactions.

It appears from the experiment that substance B while reacting strongly *in vitro* (Table I) had little if any antigenic activity.

#### SUMMARY.

The investigations described show that it is possible to extract specific substances from *V. cholerae* by means of hot dilute alcohol. This result cannot be attributed to the presence of bacilli in the extracts as would follow from the statements made by Prausnitz in his criticism of the work of Levaditi.

The original extract contains protein and exhibits antigenic properties. From this preparation an almost protein-free product was obtained. It was fully active in the precipitin test but had lost almost completely the antigenic activity. Accordingly the latter preparation belongs to the class of substances described by Zinsser as residue antigens and studied chemically by Avery and Heidelberger. The fact that the immune sera resulting from the injections of the crude extracts acted upon the non-immunizing precipitable substance indicates that in the crude extract there is present an antigenic complex consisting of protein and the specific substance.

Regarding its chemical nature it follows from the foregoing that the precipitable but non-immunizing substance is not a protein. On hydrolysis it yielded a considerable quantity of sugar although less than that given for the specific carbohydrates of pneumococci and *B. friedländeri*. The product contains nitrogen and phosphorus and on hydrolysis a substance of acid character separates from the solution. Accordingly the substance prepared would appear to have either a rather intricate structure or to be a complex carbohydrate, similar to those described by Avery and Heidelberger, but still containing impurities. This issue can probably be decided by further studies.

#### BIBLIOGRAPHY.

1. Levaditi, C., and Mutermilch, S., *Compt. rend. Soc. biol.*, 1908, lxiv, 406, 844, 1151; lxxv, 26.
2. Prausnitz, C., *Centr. Bakt., I. Abt., Orig.*, 1911, lix, 434.





## STUDIES IN EXPERIMENTAL SYPHILIS.

### VII. REINOCULATION OF TREATED AND UNTREATED SYPHILITIC RABBITS WITH HETEROLOGOUS STRAINS OF *TREPONEMA PALLIDUM*.\*

By ALAN M. CHESNEY, M.D., CHARLES R. L. HALLEY,† M.D., AND  
JAROLD E. KEMP, M.D.

(From the Department of Medicine, Johns Hopkins Medical School, Baltimore.)

(Received for publication, May 10, 1927.)

Previous communications in this series have dealt with the results of experiments in which both treated and untreated syphilitic rabbits were inoculated a second time with homologous strains of *Treponema pallidum*. The results of those experiments are in close accord with the findings of other investigators in the same field,<sup>1</sup> and the evidence thus far assembled, both by others and by ourselves, indicates that in rabbits during the course of an experimental infection with syphilis there is built up in time a specific state of resistance against the infecting organism to the extent that subsequent inoculations with *homologous* strains of the latter are not followed by the customary phenomena of the disease. It is possible to prevent the development of this state of resistance by suitable treatment early in the course of the disease (before the 45th day), but if treatment be postponed until the resistant state is fairly well established (after the 90th day), as was first shown by Kolle, the rabbit apparently remains refractory, for months at least, to a second inoculation with the homologous strain of *treponemata*, that is to say, refractory in the sense that reinoculation is not followed by any manifest phenomena of disease. It has been established in experiments already reported in this series (1) that this resistant

\* Aided by a grant from the Ella Sachs Plotz Foundation for the Advancement of Scientific Investigation.

† Jacques Loeb Fellow in Medicine.

<sup>1</sup> The present status of our knowledge of this subject has been reviewed recently by one of us in another periodical (18), and need not therefore be presented here in detail.

state against homologous strains of the organism is not absolute but may be broken down in part by resort to procedures which favor the inciting agent.

The purpose of the present communication is to record the results of a series of experiments in which the reaction of treated and untreated syphilitic rabbits toward second inoculations with *heterologous* strains of *Treponema pallidum* was observed. Since these studies were first undertaken in the latter part of 1924, there have appeared in the literature reports of experiments conducted along similar lines with results, in general, similar to those obtained by ourselves. There are also available in the older literature accounts of experiments of like character in which a few animals were studied. These will be briefly summarised at this point, attention being directed principally to those experiments in which the second inoculation was carried out 90 days or more after the first, or in which treatment was begun after a like interval had elapsed following the first inoculation.

#### HISTORICAL.

Zinsser, Hopkins and McBurney (2) inoculated 12 untreated syphilitic rabbits with heterologous strains of *Treponema pallidum*, 106 to 389 days after the first inoculation, and obtained 3 positive results. Pearce and Brown (3) were apparently able to produce a second infection in an untreated syphilitic rabbit by the inoculation of a more virulent strain of treponemata 55 days after the first inoculation, at a time when the disease phenomena produced by the latter were subsiding. Reiter (4) obtained 4 successful second infections in 9 reinoculations of rabbits with heterologous strains carried out 133 to 602 days after the first inoculation, and Kolle (5) in a series of cross-inoculation experiments with 51 rabbits obtained 23 successful second infections with heterologous strains. Adachi (6) successfully infected 7 syphilitic rabbits a second time by inoculating with heterologous strains late in the course of the disease, while reinoculations with homologous strains gave uniformly negative results. Nothhaas (7) has recently reported 3 successful reinoculations with heterologous strains in a series of 16 syphilitic rabbits, the interval between inoculations ranging from 14 to 42 weeks. Manteufel and Worms (8) carried out a series of cross-inoculations in which 7 rabbits infected with the Nichols strain and untreated were subsequently inoculated with another strain of *T. pallidum*. Of the 7, 2 were successfully infected a second time.

The behavior of *treated* syphilitic rabbits toward a second inoculation with heterologous strains of treponemata has received scarcely any attention thus far. Reiter (4) treated 3 syphilitic rabbits 70, 91 and 182 days, respectively, after

inoculation and subsequently reinoculated them with heterologous strains but failed to obtain any evidence of infection. Manteufel and Worms (8) reinoculated one treated rabbit with an heterologous strain of *T. pallidum* with a negative result.

#### EXPERIMENTAL.

The observations to be reported in this communication deal with a series of reinoculations in 68 rabbits, most of which were treated with arsphenamine prior to the introduction of the second infection.

*Rabbits.*—All the commoner breeds of rabbits were represented in the experiments, the grays and browns predominating.

*Mode of Inoculation.*—First inoculations were invariably made into the testis and in nearly every instance subsequent inoculations were made in the opposite testis. In a few instances second inoculations were made intracutaneously into the sheath of the penis. Because of the occurrence of metastatic orchitis in many of the animals, it happened that frequently the second inoculation was made in a testis which had previously been the site of a syphilitic process, while in the others the second inoculation was made into an apparently normal testis as judged by clinical examination. In some instances the animals were reinoculated first with the homologous strain, later with an heterologous strain of *treponemata*.

*Strains.*—Six strains of *Treponema pallidum* were used in this study. These included two which have long been adapted to the rabbit, namely the Nichols strain and the Truffi strain (for the latter of which we are indebted to Professor Kolle). The remaining 4 strains were isolated by us from syphilitic patients in the Johns Hopkins Hospital. Of these latter, 1, designated "A," was isolated from the spinal fluid of a patient with secondary syphilis, but without clinical or serological evidence of involvement of the central nervous system (9); 1, designated "C," was isolated from the spinal fluid of a patient with syphilitic meningitis of the neurorecurrence type (10), a third, designated "F," was isolated from the synovial fluid of a patient with syphilitic arthritis; and the fourth, Strain "H," was obtained from a lymph node of a patient with arthritis (11). All of these 4 strains were isolated in the years 1923 and 1924.

*Treatment.*—Where the animals were treated, the mode of treatment was invariably the same and consisted in the administration of 6 doses of arsphenamine, 10 mg. per kg., administered at weekly intervals. The time at which treatment was begun varied somewhat but was always late in the course of the infection, that is to say, after the 90th day. This time interval was selected because of the now well established fact that when treatment is postponed until this interval has elapsed a second inoculation with homologous strains of *Treponema pallidum* does not, except in rare instances, lead to the development of a syphilitic lesion at the site of inoculation.

*Wassermann Reaction.*—In many of the experiments the behavior of the Wassermann reaction was followed at regular intervals. The technic employed

has already been described elsewhere (12). Further experience with the method has confirmed our previous impression that it can be successfully employed in the study of experimental syphilis in the rabbit. The only modification which we have introduced since the publication of our previous communication on the test, and have employed throughout in these experiments, has been the use of 0.05 cc. of the serum instead of 0.1 cc. as originally employed. All other quantities of reagents have been the same. We have gained the impression that with the use of the smaller amount of serum a number of anticomplementary reactions have been eliminated.

*Criteria of Reinfection.*—In all the experiments cited in the literature, the criterion of successful production of a second infection has been the development, after an appropriate incubation period, of a syphiloma at the site of inoculation in which the presence of *Treponema pallidum* could be demonstrated. As was suggested several years ago by Brown and Pearce (13), however, second infections might be produced without the occurrence of any visible syphilitic phenomena at the site of reinoculation, and in previous papers in this series (14, 1) evidence has been presented which strongly indicates that treated syphilitic rabbits can react in such a manner to a second inoculation with homologous strains of the organism. Data of a similar nature have also been obtained by Voegtlin and his associates (15). Recently Kolle and Schlossberger (16) reported experiments which indicate that the same state of affairs may obtain when heterologous strains are employed for the second inoculation. The recognition of such asymptomatic reinfections is made possible through the study of the infectivity of lymph nodes or internal organs of the reinoculated animal. The utilisation of this method of study necessarily entails the expenditure of a large number of animals and in the present study it was deemed advisable to forego this procedure since the object was not to obtain absolute figures as to the susceptibility of syphilitic rabbits to infection with heterologous strains, but to contrast the effect of reinoculation with the latter as opposed to reinoculation with homologous strains. For that reason we adopted as a criterion of successful second infection in this series of experiments, the development of a characteristic syphilitic lesion at the site of inoculation with the demonstration of treponemata therein. It is freely admitted that such a criterion will probably fall short of telling the whole story, and that the number of successful second infections in our experiments may have been, and almost certainly was, greater than the figures would indicate, but the expense attendant upon ascertaining the actual number of asymptomatic reinfections, involving as it does the identification of the strain isolated after reinoculation, did not seem to be warranted in view of the fact that only relative and not absolute data were desired.

*Controls.*—The virulence of the strain used for reinoculation was, of course, always tested by simultaneous inoculation of a series of normal rabbits with equal amounts of the same virus emulsion. In no instance did the control rabbits fail to show characteristic syphilitic lesions. A series of 13 animals was twice reinoculated with homologous strains of treponemata in order to ascertain

whether or not the resistance to the homologous strain persisted for the duration of the experiments.

### *Results in Untreated Rabbits.*

Reinoculations with an heterologous strain of *Treponema pallidum* were carried out in 11 untreated syphilitic rabbits, the second inoculation in each instance being performed with the Nichols strain. The interval between inoculations varied from 93 to 170 days. In 5 of the animals the second inoculation was made intracutaneously on

TABLE I.

*Reinoculations of Untreated Syphilitic Rabbits with Heterologous Strains.*

Rabbit No.	Strain of 1st inoculation	Strain of 2nd inoculation	Interval between inoculations	Mode of 2nd inoculation	Result
			days		
1	A	Nichols	107	Testicular	—
2	C	"	93	"	+
3	C	"	93	"	+
4	C	"	93	"	+
5	C	"	107	"	+
6	F	"	170	Intracutaneous	—
7	F	"	142	"	+
8	F	"	107	"	+
9	H	"	153	"	+
10	Truffi	"	121	"	—
11	"	"	121	Testicular	—
Total positive.....					7
" negative.....					4

the sheath of the penis. In 4 of the remaining 6 animals, in which the second inoculation was made into the testis, that particular organ had previously been the site of a syphilitic lesion. The results of the experiment are shown in Table I.

Table I shows that of the 11 untreated syphilitic rabbits reinoculated with an heterologous strain of *Treponema pallidum* 93 to 170 days after the first inoculation, 7, or 63 per cent, reacted with the formation of a syphilitic lesion at the site of inoculation. In 9 of the animals the strains used for the first inoculation had been isolated from human cases of syphilis at a more recent date than had the strain used

TABLE II.

*Reinoculations of Treated Syphilitic Rabbits with Homologous and Heterologous Strains of T. pallidum.*

Rabbit No.	Strain of 1st inoculation	Treatment days after inoculation	2nd inoculation			3rd inoculation		
			Strain	Day of disease	Result	Strain	Day of disease	Result
12	A	139	A	282	—	Nichols	339	+
13	A	139	A	282	—	"	339	+
14	A	170	A	267	—	"	381	—
15	A	198	A	295	—	A	429	—
16	A	170	A	267	—	A	401	—
17	C	188	C	271	—	Nichols	388	+
18	C	169	C	238	—	"	380	—
19	C	115	C	184	—	"	326	+
20	C	206	C	275	—	C	431	—
21	C	169	C	238	—	C	394	—
22	F	213	F	326	—	Nichols	413	+
23	F	138	F	230	—	"	317	+
24	F	157	F	240	+	"	468	—
25	F	124	F	207	+	"	335	—
26	F	124	F	207	—	"	335	—
27	F	157	F	240	—	F	382	—
28	F	208	F	291	—	F	433	—
29	H	210	H	344	—	Nichols	410	+
30	H	141	H	275	—	"	341	+
31	H	265	H	348	—	"	476	—
32	H	210	H	293	—	"	421	—
33	H	157	H	240	—	H	394	—
34	H	120	H	203	—	H	357	—
35	H	120	H	203	—	H	357	—
36	Nichols	91	Nichols	160	—	A	265	—
37	"	91	"	160	—	A	265	—
38	"	116	"	185	—	A	290	—
39	"	91	"	160	—	F	265	+
40	"	91	"	160	—	F	265	+
41	"	116	"	185	—	F	290	+
42	"	91	"	160	—	F	265	+
43	"	116	"	185	—	Nichols	290	—
44	"	116	"	185	—	"	290	—
45	"	91	"	160	—	"	265	—
46	"	91	"	160	—	"	265	—

\* Lesion produced was a small syphilitic nodule.

for the second inoculation, and in general could be regarded as less virulent strains than the latter. There was no significant difference in percentages of successful reinoculations obtained by intratesticular inoculation as contrasted with intracutaneous inoculation.

### *Results in Treated Rabbits.*

For the purpose of presentation, the experiments with treated rabbits have been arranged in two groups, first (Group 1) those in which the animals were reinoculated with the homologous strain and then with an heterologous strain, and those (Group 2) in which only one reinoculation and that with the heterologous strain was carried out.

TABLE III.

*Possible and Actual Results of Reinoculations with Homologous and Heterologous Strains of T. pallidum.*

Category	1st reinoculation		2nd reinoculation		No. of rabbits encountered
	Strain	Result	Strain	Result	
A	Homologous	+	Homologous	+	0
B	"	-	"	+	0
C	"	+	"	-	0
D	"	-	"	-	13
E	"	+	Heterologous	+	0
F	"	-	"	+	12
G	"	+	"	-	2
H	"	-	"	-	8
Total.....					35

Group 1. In this group there were observations upon 35 animals including 13 controls which received two successive reinoculations with the homologous strain. The results are shown in Table II.

From Table II it will be seen that of 35 rabbits originally inoculated with various strains of *T. pallidum* and treated from 91 to 265 days after inoculation all but 2 gave negative results when inoculated a second time with the homologous strain. Of the 33 animals which were thus shown to be refractory to their own strain, 13 were inoculated a third time with the same strain as controls and again proved refractory, while the remaining 20 which were inoculated with heterologous



strains, gave a positive result in 12 instances, or 59 per cent. The two animals (Nos. 24 and 25) which proved to be susceptible to a second inoculation with their own strain were subsequently inoculated with an heterologous strain and proved to be refractory to the latter, rather to our surprise. We have no satisfactory explanation for this result.

The foregoing results may perhaps be better appreciated if one considers all the theoretically possible combinations and contrasts them with the results actually obtained. If syphilitic rabbits are twice reinoculated, first with the homologous strain and then with either the same or another strain, it is apparent that there are 8 possible results to such an experiment. In Table III these are listed together with the number of rabbits encountered in each category.

TABLE IV.

*Reinoculation of Treated Syphilitic Rabbits with Heterologous Strains.  
Treatment 122 Days after Inoculation.*

No. of rabbits	Strain of 1st inoculation	Strain of 2nd inoculation	Result		Per cent positive
			Positive	Negative	
11	Nichols	A	2	9	18
11	"	F	7	4	63

From consideration of Table III it is seen that of the 8 theoretically possible results 4 were actually encountered in the experiments. The tendency of syphilitic rabbits treated late in the course of the disease to react to a second inoculation with the homologous strain in such a manner as to exhibit no evidence of a lesion at the site of inoculation is clearly shown in the table, and is in striking contrast with their behavior when reinoculated with heterologous strains.

Group 2. In this group there were observations upon 22 rabbits all of which were inoculated at the same time with the Nichols strain, were treated on the 122nd day of the disease and subsequently reinoculated with either Strain A or Strain F on the 197th day after the original infection. The results are shown in Table IV.

Study of Table IV shows that of 11 syphilitic rabbits inoculated with the Nichols strain and treated 122 days after inoculation, only 2,

or 18 per cent, showed lesions at the site of inoculation when inoculated subsequently with Strain A, whereas of 11 similar rabbits inoculated subsequently with the F strain 7, or 63 per cent, reacted with the development of a characteristic syphilitic lesion at the site of inoculation. In short, infection with the Nichols strain under the conditions of the experiment appeared to protect a much higher percentage of animals against infection with Strain A than against infection with Strain F. This rather marked difference in the reaction of the "Nichols rabbits" to two different strains can scarcely be attributed to differences in the size of the inoculum, since, as a matter of fact, in the group inoculated with the A strain the inoculum was considerably richer in treponemata than in the group inoculated with Strain F, in which a much higher percentage of positive results was obtained.

TABLE V.

*Cross-Inoculations with Heterologous Strains of Treponema pallidum.*

No. of rabbits	Strain of 1st inoculation	Strain of 2nd inoculation	Result		Per cent positive
			Positive	Negative	
14	Nichols	A	2	12	14
4	A	Nichols	2	2	50
15	Nichols	F	11	4	73
8	F	Nichols	4	4	50

Indeed there is no evidence at present that variations in the size of the inoculum influence the response of syphilitic rabbits to a second inoculation, although it is possible that they may.

A more plausible explanation of the difference in the behavior of the two heterologous strains would be the assumption that a closer biological relationship existed between the Nichols strain and Strain A than between the former and Strain F. The validity of such an assumption would be strengthened if it could be shown that animals infected with Strain A were protected to a greater extent against subsequent infection with the Nichols strain than were those infected with Strain F. Unfortunately our experiments do not clear up this point since they contained too few animals upon which to base conclusions. However since it may be of interest to determine to what extent they do throw

light upon this matter, we have assembled all the cross-inoculation experiments conducted with these two strains and the Nichols strain. They are shown in Table V.

As will be seen from Table V, the percentage of successful second infections obtained when rabbits infected with the Nichols strain were subsequently inoculated with Strain A was much less than when Strain F was used for reinoculation, whereas when animals infected with Strain A or Strain F were subsequently inoculated with the Nichols strain the percentage of successful second infections was the same. In other words, although the Nichols strain appeared to protect against infection with Strain A to a much greater extent than against Strain F, nevertheless infection with Strain A did not, in this limited number of observations, appear to protect against the Nichols strain to a greater extent than did infection with Strain F. Cross-protection was not complete, therefore, and although the number of animals studied was perhaps too small to warrant generalisations, the fact that it was not complete suggests that if a biologic relationship exists between Strain A and the Nichols strain it is at best one-sided under the conditions of the experiment.

One other possible explanation of the results recorded in Table V should be considered, namely, whether relative differences in virulence of Strains A and F would account for the differences in percentage of successful reinoculations obtained with these strains. It was suggested by Pearce and Brown (3) that relative differences in virulence of strains of *Treponema pallidum* might explain superinfection or reinfection with heterologous strains, and Nothhaas (7) has recently taken the same ground. If this is the correct and sole explanation for the differences in behavior of the two strains, A and F, when inoculated in rabbits previously infected with the Nichols strain, it follows that Strain F is more virulent than Strain A, and it may be said that our experience with these two strains, extending over a period of 3 years or more, tends to confirm that conclusion. On the other hand the behavior of the rabbits first infected with Strain A or Strain F and subsequently inoculated with the Nichols strain does not support that conclusion, since the percentage of successful reinoculations with the latter strain was the same in each group. However, since the number of animals in these groups was rather small we prefer to suspend judgment in

the matter until more data have been accumulated. It is possible, of course, that biologic relationship between strains of *Treponema pallidum* and relative differences in their virulence may each be a factor in determining the outcome of reinoculation experiments in which heterologous strains are employed, and in that case it may be very difficult if not impossible to ascribe to each factor its relative importance. However it is clearly shown by these reinoculation experiments that rabbits infected with a strain of *T. pallidum* which has been adapted to that species for a period of 12 years may, in a high percentage of instances, be infected a second time with other strains which have been recovered recently from the human body and have not had opportunity over so long a period for adaptation to the rabbit.

As has already been stated, the criterion of production of a successful second infection in these experiments has been the development of a characteristic lesion at the site of inoculation coupled with the demonstration of treponemata in the lesion. Using this criterion and taking into consideration all the experiments, the number of successful reinoculations obtained with heterologous strains amounted to 28 in 56, or 50 per cent, whereas the total number of successful reinoculations with homologous strains was 2 in 35, or 5.4 per cent. That this criterion is inadequate in that it would fail to disclose an asymptomatic reinfection ("stumme" infection of Kolle) has been admitted. If such asymptomatic reinfections occurred in the experiments they could not have been recognised since, for reasons already stated, we were unable to determine the infectivity of the lymph nodes and internal organs of animals in which no lesions developed following reinoculation with heterologous strains. However the behavior of the Wassermann reaction in a number of the rabbits which, upon clinical grounds, were regarded as not having been successfully reinfected, led us to suspect that a number of these animals had in reality been reinfected with heterologous strains, even though no lesions developed at the site of reinoculation. In order to show the grounds for this suspicion we have thought it wise to record the behavior of the Wassermann reaction in a number of treated animals reinoculated with heterologous strains, where the test was performed repeatedly upon the serum of the same animal over a period of weeks following reinoculation. These data are shown in Table VI.

TABLE VI.

*Behavior of Wassermann Reaction in Treated Syphilitic Rabbits Reinoculated with Heterologous Strains of T. pallidum.*

Rabbit No.	Wassermann reaction						Result of reinoculation	
	Days after reinoculation						Clinical lesion	Time, days after reinoculation
	19	33	48	77	90	105		
36	0	Ac	Ac	Ac	D		—	—
37	0	0	0	1	4	4	—	—
38	0	0	0	0	0	0	—	—
39	0	0	Ac	4	4	4	+	46
40	0	0	4	3	1	1	+	30
41	0	0	4	4	4	D	+	38
42	0	0	4	3	4	0	+	22
47	0	0	0	0	1	0	—	—
48	0	0	1	0	1	0	—	—
49	0	0	0	0	0	0	—	—
50	0	0	0	4	4	D	+	?
51	0	0	0	0	3	0	—	—
52	0	0	0	1	1	0	—	—
53	0	0	0	0	0	0	—	—
54	0	0	0	0	0	0	—	—
55	0	4	4	4	4	4	—	—
56	2	4	4	4	4	4	+	30
57			0		0	0	—	—
58	0	0	1	2	4	D	+	?
59	0	Ac	Ac	4	4	4	—	—
60	0	0	Ac	4	D		+	45
61	0	0	0	4	4	D	—	—
62	0	0	Ac	4	4	D	—	—
63	0	2	4	4	D		+	30
64	0	1	3	4	4	4	—	—
65	0	Ac	4	4	4	4	+	30
66	0	2	4	D			+	57
67	0	0	4	4	4	2	+	30
68	0		Ac	1	1	0	+	22

0—negative Wassermann.

1—25 per cent fixation.

2—50 per cent fixation.

3—75 per cent fixation.

4—100 per cent fixation.

Ac—anticomplementary.

D—died or discarded.

A consideration of Table VI shows that in 13 of 29 rabbits inoculated first with the Nichols strain, then treated and subsequently reinoculated with heterologous strains (Strain A or F) a characteristic syphilitic lesion developed at the site of reinoculation. In all but 1 of these 13 animals the Wassermann reaction, which before reinoculation had been negative, became completely positive about the time of appearance of the lesion associated with the second infection. In the 16 rabbits in which no lesion developed at the site of reinoculation the test, which at first was negative, became completely positive in 6 animals (Nos. 37, 55, 59, 61, 62 and 64), complete fixation being obtained upon at least 2 or more occasions. We are strongly inclined to the view that these animals were in reality successfully reinoculated, for in former experiments dealing with reinoculations with homologous strains (1) a close parallelism was found between infectivity of lymph nodes and the occurrence of a positive Wassermann reaction in rabbits in which no lesion developed at the site of reinoculation. Of the remaining 10 animals the serum of 1 (No. 51) gave 75 per cent fixation upon one occasion only and the sera of the other 9 gave repeated negative tests or at most only 25 per cent fixation, or were anticomplementary.

One more point may be considered, namely, what influence was exerted upon the occurrence of reinfections by the previous existence of syphilitic lesions in the area where the reinoculations were made. Zinsser (17) was of the opinion that reinoculation of a testis that had previously been the site of a syphilitic inflammatory process was less apt to yield a positive result than if the testis had not previously been involved. According to him a syphilitic lesion when it healed might leave the tissues in that area more highly resistant to second infection than other parts that had not been the seat of syphilitic inflammation. Our experiments do not bear out this conception since many successful reinoculations were obtained with heterologous strains in instances where testes were inoculated which had previously been extensively involved in the syphilitic reaction. In general, where successful second infections were obtained with heterologous strains, the character of the lesion produced was not greatly different from that of the controls. It is true, however, that in some of the animals the lesion associated with the second infection appeared earlier and was of the

abortive type, as if the preceding infection had conferred a slight resistance upon the animal, but in the majority it was fully comparable to that seen in the controls. In a few also the incubation period was prolonged by a few days but the difference in this respect was neither constant nor striking.

#### SUMMARY AND CONCLUSIONS.

Syphilitic rabbits, whether untreated or treated after the 90th day of infection, were found to be more refractory to subsequent inoculation with the homologous strain of *Treponema pallidum* than to inoculation with heterologous strains of the same organism, when clinical criteria alone were employed in judging the outcome of reinoculation. The incidence of second infection with homologous strains was 5.4 per cent, as against 50 per cent with heterologous strains.<sup>2</sup> The resistance which develops in rabbits during the course of a syphilitic infection appears therefore to be strain-specific rather than species-specific. The protection afforded against homologous strains was found to persist for at least as long as 6 months after treatment was discontinued.

A given strain may afford a higher degree of protection against some strains than against others, but whether this is to be explained upon the basis of biologic relationship or of differences in virulence, or possibly as the result of both of these factors was not disclosed by the experiments. Rabbits infected with a strain (Nichols) which had been adapted to this species for over a decade could be infected with strains which had been recovered recently from the human body. The previous existence of a syphilitic lesion in the testis which was used as the site for reinoculation did not seem to exert any influence upon the incidence of successful second infections obtained with heterologous strains of *Treponema pallidum*. Sometimes the course of the second infection produced by inoculation with heterologous strains

<sup>2</sup> In this respect it is of interest to call attention to some recent inoculation experiments upon untreated syphilitic human beings, carried out by Hashimoto (19). This observer noted a higher percentage of positive results when patients with untreated syphilis were inoculated with syphilitic virus from other patients than when inoculated with their own virus.

was less pronounced than that observed in the controls, but in most instances no significant alteration was observed.

In syphilitic rabbits treated late in the course of the disease and reinoculated with heterologous strains of *Treponema pallidum* no lesion may develop at the site of reinoculation but nevertheless the Wassermann reaction may become positive and remain so for weeks thereafter. It is suggested that such animals may be examples of asymptomatic reinfection.

#### BIBLIOGRAPHY.

1. Chesney, A. M., and Kemp, J. E., *J. Exp. Med.*, 1926, xliv, 589.
2. Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, xxiv, 561.
3. Pearce, L., and Brown, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 258.
4. Reiter, H., *Centr. Bakt., 1. Abt., Orig.*, 1924, xcii, 534.
5. Kolle, W., *Deutsch. med. Woch.*, 1926, lii, 11.
6. Adachi, Y., *Acta Dermatol. (Japan)*, 1925, v, 42.
7. Nothhaas, R., *Deutsch. med. Woch.*, 1927, liii, 102.
8. Manteufel, P., and Worms, W., *Centr. Bakt., 1. Abt., Orig.*, 1927, cii, 23.
9. Chesney, A. M., and Kemp, J. E., *J. Am. Med. Assn.*, 1924, lxxxiii, 1725.
10. Kemp, J. E., and Chesney, A. M., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 199.
11. Chesney, A. M., Kemp, J. E., and Resnik, W. H., *Bull. Johns Hopkins Hosp.*, 1924, xxxv, 235.
12. Kemp, J. E., Chesney, A. M., and Poole, A. K., *Bull. Johns Hopkins Hosp.*, 1926, xxxix, 132.
13. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 255.
14. Chesney, A. M., and Kemp, J. E., *J. Exp. Med.*, 1925, xlii, 17.
15. Voegtlin, C., and Dyer, H. A., *Pub. Health Rep., U. S. P. H.*, 1925, xl, 2511.
16. Kolle, W., and Schlossberger, H., *Deutsch. med. Woch.*, 1926, lii, 1245.
17. Zinsser, H., *Infection and resistance*, New York, 3rd edition, 1923.
18. Chesney, A. M., *Medicine*, 1926, v, 463.
19. Hashimoto, T., *Bruxelles-méd.*, 1926, vi, 1362.





## STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

### VII. THE RELATION OF OPSONINS TO NATURAL RESISTANCE AGAINST PNEUMOCOCCUS INFECTION.\*

By OSWALD H. ROBERTSON, M.D., AND RICHARD H. P. SIA, M.D.

*(From the Department of Medicine, Peking Union Medical College,  
Peking, China.)*

(Received for publication, April 25, 1927.)

It seems not improbable that the filling in of some of the many gaps in our knowledge of the mechanism of recovery from bacterial disease and acquired immunity may depend primarily upon a clearer understanding of the processes by which the naturally resistant animal defends itself against pathogenic microorganisms. Information concerning the nature of these processes is as yet largely general in character. Although it has been long recognized that the body is capable of certain well defined antibacterial responses, the exact rôle they play in the destruction of the different species of invading bacteria remains for the most part unknown. The search for immunity factors underlying natural resistance has proved particularly baffling as reactions shown characteristically by the body cells or fluids, following recovery from an infection, have been found to be absent or to occur only irregularly in the naturally immune animal.

A review of the literature on natural immunity to the pneumococcus reveals clearly this lack of a uniform, or even at times detectable, response by the pneumococcus-resistant body. The reaction observed to occur most constantly in studies on this subject is phagocytosis, but while certain workers have found that virulent pneumococci injected into the tissues of pneumococcus-resistant animals were promptly and constantly engulfed by the leucocytes (1, 2), others, apparently employing similar experimental conditions, have noted phagocytosis to be variable (3) or even absent (4). Furthermore,

\* Read before the Sixth Congress of the Far Eastern Association of Tropical Medicine, held in Tokyo, Japan, October, 1925.

doubt has been cast on the assumption that phagocytosis is to be considered the chief, or perhaps even a very important, means of antipneumococcus defense, by the lack of any evidence to show that the serum of resistant animals possesses greater phagocytosis-promoting power or that their leucocytes are more actively phagocytic than the serum and leucocytes of susceptible animals. Indeed highly virulent pneumococci have been found to be uniformly resistant to phagocytosis *in vitro* except in the presence of specific antipneumococcus serum (5-8). Attempts to protect susceptible animals against infection with the serum of highly resistant mammals have not been successful.

The failure to find humoral defense substances in the resistant animal's blood and the inconclusive results of the observations on phagocytosis led investigators to test the action of leucocyte extracts on the pneumococcus, since it seemed certain that the leucocytes must play a rôle in the defense against this organism. It was found that pneumococci seeded into extracts of leucocytes failed to grow and were either reduced in numbers or disappeared entirely (9-11). Furthermore, leucocyte extracts injected during the course of experimental pneumococcus infection were considered to have influenced the course of the disease favorably (12). Pointing toward the primary importance of cellular activity in the antipneumococcus defense mechanism is the further observation that in the pigeon certain of the large fixed tissue cells play an active part in removal by phagocytosis of injected pneumococci (13). The above findings have given rise to the view quite widely held that natural immunity to the pneumococcus, in common with other pathogenic bacteria, depends on certain fundamental characteristics of the body cells and not on circulating antibodies (14-16).

However, of recent years evidence has been accumulating to indicate that natural resistance to pneumococcus infection may depend much more on humoral defense elements than was formerly thought.

As long ago as 1910 Neufeld and Händel (17) found that the blood serum of some normal humans protected mice against otherwise fatal doses of pneumococci. Cecil and Austin (18) showed a protective action in the serum of one out of sixteen normal humans tested. Recently Clough (19) in an extensive study of the protective properties in normal human serum of individuals without antecedent

history of pneumonia,<sup>1</sup> demonstrated definite protective power in 11 of 22 sera tested, against one or more of the three fixed types of pneumococcus. The degree of protective power was usually slight but in five instances the serum protected against from 1000 to 100,000 M.L.D. He failed to find opsonic or agglutinative action in any of these sera. Burhans and Gerstenberger (20) studied the anti-pneumococcal protective power of both mothers' and infants' blood serum and found protective properties in 30 to 40 per cent of more than 100 cases tested. Bull and McKee (21) in 1921 showed that chicken serum conferred on mice well marked protection against many times the lethal dose of highly virulent pneumococci of all four types. They were able to isolate the protective substance from the serum in the water-insoluble globulin fraction. In a study of the mechanism of natural immunity in the dog, Bull (22) found that highly virulent pneumococci injected into the blood stream were agglutinated and carried to the liver and spleen principally. He was unable, however, to demonstrate *in vitro* either agglutinins or opsonins in the dog's serum.

Thus while the findings summarized above show that here and there among the pneumococcus-resistant animal species certain body reactions against the pneumococcus have been detected, they give no indication that there exists a common means of protection, or anti-pneumococcus reactive process, even among the mammals. Our finding, reported in an earlier paper (23), of a constantly demonstrable pneumococcidal action in the blood of the dog and the cat for highly virulent strains which the blood of susceptible animals failed to show, together with Woo's (24) demonstration of a similar action in rabbits' blood against pneumococci of low virulence for the rabbit, suggests that animals naturally immune to pneumococcus infection do possess a common means of defense against this organism. In previous studies no attempt was made to determine the exact nature of the pneumococcus-destroying power of the blood further than to observe that this property did not reside in the serum alone nor in the leucocytes with inactivated serum. It appears to depend on the combined action of the fresh serum and leucocytes. It was the purpose of the present investigation to study the mechanism of this reaction and to determine if possible whether the differences in blood pneumococcidal activity shown by the various animal species depend on differences in the serum constituents or on the character of the leucocytes.

<sup>1</sup> One person gave a history of pneumonia many years previously.

*Technique.*

The basis of the technique employed in carrying out the phagocytic tests was that originally described by Wright and Douglas (25). It was found necessary, however, to modify this procedure, particularly in regard to two points—the preparation of the leucocytes and the growth phase of the organisms.

*Leucocytes.*—The leucocytes employed in the following experiments were obtained from aleuronat exudates withdrawn from the pleural cavity 15 to 18 hours after injection of the aleuronat. The solutions used for suspension and washing were the same as those employed in the former work with growth inhibition tests (23). After the second washing in gelatin-salt solution the leucocytes were suspended in gelatin-Locke's solution and were ready for use. A standard suspension was made such that each c.mm. contained 50,000 white blood cells. In order to prevent clumping of the leucocytes it was found necessary to mix red blood cells with them. After the leucocytes had been counted, and before the first centrifugation, 0.75 to 1.5 cc. of a mixture of equal parts of blood and 1 per cent citrate-0.9 per cent salt solution were added to the suspension, the quantity added depending on the richness of the exudate. Centrifugation was carried on at low speed and for as short a time as necessary to completely sediment the corpuscles.

All glassware with which the leucocytes came into contact was cleaned with especial care, according to the technique described in the former publication referred to. The leucocytes were always used fresh and were kept on ice until actually employed in the pipette mixtures.

*Organisms.*—The Type I pneumococcus employed in Experiments 1 to 5 was a strain originally isolated from a patient with lobar pneumonia and kept for a number of years in blood broth with an occasional animal passage. Immediately before the beginning of the present work it was passed rapidly through ten rabbits and its virulence for rabbits, guinea pigs, dogs and cats tested with the following results: 0.0000001 cc. killed 1500 to 1600 gm. rabbits in 24 to 36 hours; 0.000001 cc. killed guinea pigs of 470 gm. in 15 days; 2.5 cc. to 5 cc. killed dogs weighing from 15 to 20 kilos in 24 to 48 hours; 1 cc. to 5 cc. killed cats of 2000 to 2500 gm. in 48 hours.<sup>2</sup> During the progress of the work this strain was passed through a rabbit approximately once a month in order to eliminate avirulent members which might appear in the culture.

The Pneumococcus Type II used in Experiment 6 was a strain secured from The Rockefeller Institute, originally isolated from a case of lobar pneumonia and designated as D 39. Even after passage through a series of rabbits, its virulence for this animal remained low—0.1 to 0.01 cc. was required to kill rabbits of 1500 to 1800 gm.

---

<sup>2</sup> This was the most virulent organism we had in our possession. Other strains of Types II, III and IV were much less virulent and hence less suitable for the purposes of this work.

The growth phase, media employed and suspension of pneumococci for use will be dealt with in the first two experiments.

*Sensitization.*—After thorough mixture of pneumococcus suspension with serum in a 15 cc. centrifuge tube, sensitization was carried on in the water bath at 37°C. for 1 hour unless otherwise stated. The organisms were then sedimented by centrifugation at high speed for 1 hour, the serum next completely removed and the pneumococci taken up in sufficient Locke's solution pH 7.6 to make a suspension somewhat more concentrated than that originally added.

*Opsonic Test.*—Wright's capillary pipettes and technique of making mixtures were used. The pipettes were sealed with paraffin and incubated for 45 minutes. After opening, the supernatant fluid was expelled gently and only a small amount used to wash out the sedimented cells. In this way a thick cell suspension was obtained. Films were made on cover-slips 22 × 35 mm. and stained with Cross's stain. To estimate the degree of phagocytosis, 100 leucocytes were counted in each preparation. The per cent of leucocytes taking part in phagocytosis, as well as the number of leucocytes containing 5 or more pairs of pneumococci, were recorded. In some experiments counts of the per cent of leucocytes containing 20 or more pairs were made. Wright's phagocytic index was not determined. In order to estimate the variation in counts from pipette to pipette, and in different preparations from the same pipette, counts were made on a number of pipettes containing the same mixture of serum, leucocytes and pneumococci. The amount of variation in the counts thus made was found to be conditioned chiefly by the degree of phagocytosis occurring. With a mixture showing marked phagocytosis, the counts from pipette to pipette were surprisingly uniform—the variation was usually not more than 5 to 10 per cent. But as the intensity of phagocytosis lessened, the differences between pipettes increased until fluctuations of 25 to 50 per cent above or below might be expected with mixtures yielding only slight phagocytosis. Relatively little difference was found between counts on duplicate preparations from the same pipette. Spontaneous phagocytosis of unsensitized pneumococci was practically absent.

#### EXPERIMENTAL.

##### *Concentration of Normal Antipneumococcus Opsonins in Dog Serum.*

Since it seemed most probable that the pneumococcidal power of the blood of naturally resistant animals was to be explained by phagocytosis and intracellular digestion, investigation of the opsonic properties of the serum and phagocytic activities of the leucocytes was undertaken first. The consistently negative results of previous investigators indicated clearly, however, that no further information could be gained by carrying out phagocytic tests in the usual way. A clue to the correct method of approach was afforded by the quanti-

tative nature of the pneumococcus-destroying action shown by the serum-leucocyte mixtures. Given a fixed amount of serum, the number of pneumococci killed never exceeded a definite maximum amount of standard pneumococcus suspension, which was quite small in comparison with the quantity of serum used. The number of leucocytes could be varied widely without influencing the result. This made it seem likely that if opsonic action played a part in the process, it could be demonstrated only by employing a relatively large ratio of serum to pneumococci. In the following experiment a fixed quantity of pneumococcus suspension was sensitized with varying amounts of dog serum and the resulting degree of phagocytosis by dog leucocytes observed.

TABLE I.

*Concentration of Normal Opsonins in Dog Serum.*

Dog-serum-sensitized pneumococci + dog leucocytes + dog serum 1:5 dilution.

Quantity of dog serum used for sensitization	Amount of pneumococcus suspension	Degree of phagocytosis	
		Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
cc.	cc.		
5.0	0.1	99	96
2.5	"	87	62
1.0	"	45	12
0.1	"	0	0
Control with unsensitized pneumococci. . . .		0	0

*Experiment 1.*—(Table I.) A normal dog weighing 16 kilos was bled for serum just before injection of aleuronat on day preceding test. The serum was kept in the ice box overnight. The organisms, cultured in 1 per cent rabbit serum broth, and having passed through the phase of active growth, were freed from their culture fluid by centrifugation, then suspended in gelatin-Locke's solution pH 7.8. The density of the suspension was approximately that of the standard suspension used in former experiments (23). Actual standardization was not made. The pneumococci sedimented from the sensitizing serum showed well marked clumping in all but the tube containing the smallest quantity of serum. The agglutinated mass could be broken up fairly easily with a capillary pipette in the gelatin-Locke's solution. However, with the largest amount of serum small clumps still remained. The phagocytosing cells were almost entirely polymorphonuclears. Only an occasional active monocyte seen.

The results of the above experiment are shown in Table I. It was found that with a ratio of sensitizing serum to pneumococcus suspension of 50 to 1, marked phagocytosis occurred. Practically all the leucocytes were packed with organisms. Diminishing the quantity of sensitizing serum resulted in a progressive decrease of phagocytosis until, with equal parts of serum and pneumococcus suspension, phagocytosis no longer occurred. A comparison of the figures under per cent of leucocytes containing five or more pairs gives a more accurate idea of the relative degree of phagocytosis than do the percentages in the first column. Repetitions of this experiment with both dog and cat serum under identical conditions gave essentially the same results.

*The Effects of Growth Phase and Culture Fluid on Opsonic Action.*

In further experiments on the opsonic action of dog and cat serum, marked variations in the degree of resulting phagocytosis were noted from test to test. These variations appeared to be greater than could be accounted for by individual differences in the animals providing the serum. It was then observed in several experiments in which the degree of phagocytosis was relatively low that the pneumococci used were in the active growth phase and continued to grow in the sensitizing serum. This led to a consideration of the effect of growth state on opsonization and phagocytosis. In the preceding paper one of us (26) showed that the soluble specific substance of the pneumococcus, both in purified form and in the filtrate of actively growing cultures, could inhibit to a marked degree the pneumococcal action of normal serum-leucocyte mixtures. The nature of this inhibiting effect was not investigated at that time but it was considered most probably to be an interference with opsonization analogous to the action of Rosenow's "virulin" (27). In spite of the fact that the organisms used in the present experiments had been separated from their growth products before use, it seemed not improbable that an actively growing highly virulent pneumococcus might elaborate, during the period of suspension in the sensitizing serum, sufficient soluble substance to protect itself partially or completely against the action of normal opsonins. The possibility also existed that the growth products of pneumococci might affect the leucocytes so as to diminish



their phagocytic activity, although the result of previous work made this seem unlikely. The next experiment was devised to test the effect of growth phase and soluble substance on the phagocytic activity of normal dog serum and leucocytes.

TABLE II.

*Effect of Growth Phase and Culture Fluid on Opsonic Activity of Dog Serum.*

Dog-serum-sensitized pneumococci + dog leucocytes + dog serum 1:5 dilution.

Growth phase	Amount of sensitizing serum	Fluid added to sensitizing serum	Time of sensitization	Fluid added to pipette mixtures	Degree of phagocytosis		
					Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs	Per cent of packed cells; leucocytes containing 20 or more pairs
	cc.						
Lag	5	0.4 cc. broth	1 hr.	—	96	91	45
Active	"	" " "	" "	—	53	10	0
Lag	"	" " "	30 min.	—	98	83	6
Active	"	" " "	" "	—	87	53	2
Lag	"	" " "	15 min.	—	90	58	10
Active	"	" " "	" "	—	76	49	1
Lag	"	" " "	1 hr.	Culture fluid equal parts	78	76	34
Lag	"	0.4 cc. culture fluid	" "	—	62	34	8
		Controls with unsensitized pneumococci					
Lag				—	1	0	0
Active				—	1	0	0

*Experiment 2.*—(Table II.) Serum and leucocytes secured as in Experiment 1 from a normal dog weighing 17 kilos. To obtain pneumococci suitable for studying the lag state, seedings were made into flasks containing meat infusion broth to a depth of 1.5 cm.<sup>3</sup> The growth phase was tested by replanting a small quantity

<sup>3</sup> The employment of a relatively large surface area of culture fluid for the purpose of constant and early cessation of active growth was suggested by Avery's work on the occurrence of peroxide in pneumococcus cultures (Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 275). A small quantity of dextrose, 0.05 per cent, was added to the broth on account of the very low sugar content of broth made from North China beef.

of the culture, freed from its fluid, into 50 per cent rabbit serum broth. Growth was easily detectable macroscopically by comparison with a control suspension kept in the ice box. It was found that 0.05 cc. of an actively growing culture seeded into 33 cc. of broth contained in a 100 cc. Erlenmeyer flask, which gave the desired depth of fluid, usually completed its active growth after 16 to 18 hours incubation. Plates of dilutions of these cultures showed that for a period of 2 to 3 hours after growth had ceased there was no appreciable diminution in the number of colonies. Several flasks were seeded the day before the test at times appropriate to give cultures of 14, 16, 18 and 20 hours. The youngest culture showing no growth in the rabbit serum broth at the end of 2 hours was chosen for the test.

The actively growing culture used was a 14 hour test-tube growth. The supernatant fluid of this culture, centrifuged until microscopically free from pneumococci, was employed in the experiment. Suspensions of the organisms were made as in the preceding experiment.

The growth phase of the organism was found to exert a marked effect on the degree of phagocytosis of pneumococci by dog serum and leucocytes (Table II). Pneumococci in the state of lag during sensitization were subsequently engulfed to a much greater extent than were the organisms which continued to grow actively during this period. The contrast between active growth and lag phase is shown most strikingly by the pneumococci sensitized for 1 hour, which gave the maximum amount of phagocytosis of the lag organisms but resulted in only a very moderate degree of ingestion of the actively growing pneumococci. The effect of growth during sensitization is further shown by a comparison of the phagocytosis percentages of actively growing organisms sensitized for varying periods of time. An hour's sensitization gave considerably less phagocytosis than did the 15 and 30 minute periods.

The addition to the sensitizing serum of a small quantity of the supernatant fluid of the actively growing culture reduced considerably its opsonic action on pneumococci in the lag phase. (Compare Pipettes 1 and 8, Table II.) That the pneumococcus soluble substance acts primarily on the opsonins and not on the leucocytes is indicated by the fact that culture fluid mixed with leucocytes and previously sensitized organisms did not retard to any extent the resulting phagocytosis. The difference between Pipettes 1 and 7 can be accounted for by the increased amount of fluid in Pipette 7, which

always lowers the phagocytosis percentage a little. In further experiments it was found that by progressively increasing the amounts of culture fluid in the sensitizing serum, opsonic action could be almost completely inhibited. Broth in equivalent amounts produced no such effect.<sup>4</sup> This result is quite in accord with Rosenow's findings.

*Phagocytosis of Pneumococci Sensitized by Alien Serum.*

Having determined those factors which influence phagocytosis so markedly in homologous serum-leucocyte mixtures, the next step was

TABLE III.

*Comparative Opsonic and Phagocytic Activity of Serum and Leucocytes of Dog and Rabbit.*

Sensitized pneumococci + leucocytes + serum diluted 1:5.

Kind and quantity of sensitizing serum	Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
				Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
Dog 5 cc.	0.1	Dog	Dog	98	94
" " "	"	Rabbit	Rabbit	69	45
Rabbit 5 cc.	"	Dog	Dog	0	0
" " "	"	Rabbit	Rabbit	1	0
Dog 5 cc. inactivated at 56°C.	"	Dog	Dog	17	1
	"	Rabbit	Rabbit	1	0
Controls with unsensitized pneumococci		Dog	Dog	0	0
		Rabbit	Rabbit	0	0

to ascertain whether the opsonins of the resistant animals sensitize the pneumococcus for phagocytosis generally, as do immune opsonins, or whether they are effective only with their own leucocytes. One of the chief difficulties met with in carrying out such tests lies in the injurious action of alien serum on the leucocytes. Hence in the following experiment in which pneumococci were sensitized with dog

<sup>4</sup> The effect of pneumococcus soluble substance on opsonic action will be taken up in detail in a further communication.

serum and tested with rabbit leucocytes, special precautions were taken to remove the serum as completely as possible from the sensitized organisms.

*Experiment 3.*—(Table III.) Serum and leucocytes from a dog weighing 24 kilos. Rabbit serum from a rabbit weighing 1650 gm. Rabbit leucocytes from another animal of the same weight. Dog serum inactivated by heating at 56°C. for 30 minutes. Pneumococci, for sensitization in the lag phase, were prepared as in the preceding experiment. After centrifugation the last portion of the supernatant serum was removed with a fine capillary pipette. Next, the surface of the sediment and the lower 2 to 3 cm. of the walls of the centrifuge tube were washed with a small quantity of gelatin-Locke's solution pH 7.4 run in slowly with the tube tilted almost horizontal. This fluid was removed with a capillary pipette and more gelatin-Locke's added for suspension. The dog-serum-sensitized pneumococci showed moderate clumping. No agglutination of the organisms suspended in the rabbit serum was seen. No hemolysis or clumping of rabbit cells was observed in the capillary pipette mixtures. Slight hemolysis and methemoglobin formation occurred in all the pipettes containing dog cells.

Virulent pneumococci sensitized by dog serum as in the above experiment were engulfed by rabbit leucocytes to a marked degree (Table III). Many of the leucocytes were packed with organisms. In striking contrast is the absence of phagocytosis of rabbit-serum-treated pneumococci by either dog or rabbit leucocytes. The failure of dog leucocytes to ingest unsensitized organisms would indicate that the behavior of these cells in the presence of unaltered virulent pneumococci does not differ essentially from that of rabbit leucocytes. The greater activity shown by dog leucocytes for the dog-serum-sensitized pneumococci may be accounted for by a certain degree of cytotoxic action of dog serum on the rabbit leucocytes. In other similar experiments analogous results have been obtained. Tests of the comparative phagocytic activity of dog and rabbit leucocytes in the presence of specific antipneumococcus horse serum, 1:200 dilution, have shown rabbit leucocytes to be fully as active as dog leucocytes. Inactivation of the dog serum largely destroyed its opsonic activity.

The growth state of the pneumococcus was found to exert a more pronounced effect on heterologous than on homologous serum-leucocyte systems. Rabbit leucocytes showed relatively little phagocytic activity for actively growing pneumococci sensitized by dog serum. That the greater phagocytability of these organisms when sensitized

during the lag phase, was not due to impaired vitality was determined by rabbit virulence tests which showed them to be quite as virulent as the actively growing pneumococci.

*Occurrence of Opsonic Activity in Sera of Cat, Sheep, Pig and Horse.*

Tests were next undertaken to determine whether the serum of other animals naturally resistant to the pneumococcus likewise possessed opsonins for this organism, and whether the difference in phagocytic activity between their serum and leucocytes and those of susceptible animals lay also in the humoral rather than in the cellular elements of the blood. For this purpose the cat, sheep, pig and horse were chosen to represent a wide variety of the resistant animals, while the human, rabbit and guinea pig were employed as typically susceptible ones.

*Experiment 4.*—(Table IV.) The data assembled in Table IV represent three separate experiments. The cat, sheep and rabbit elements were tested at one time, the pig, horse, human and rabbit at another, and the pig, guinea pig and rabbit at still a third. The animals employed were large, normal and full grown adults. The human serum was obtained from two normal individuals. Agglutination was present in all the tubes with sera of resistant animals, but none occurred in the sera of susceptible animals. No hemolysis was observed in the cross phagocytic tests. Predominantly active cells were polymorphonuclears.

The uniform occurrence in the serum of all the pneumococcus-resistant animals tested of opsonic activity that can be demonstrated not only with its own but with leucocytes of alien species, both resistant and susceptible, is strikingly shown in Table IV. The lack of phagocytic activity on the part of the leucocytes of resistant animals, for pneumococci exposed to the action of susceptible animal's serum, is equally noteworthy. The varying degrees of phagocytosis shown by the serum and leucocytes of the different animals are not to be taken as strictly comparative of the opsonic content of their sera even when tested at the same time against the same alien leucocytes because of certain variable factors which cannot be readily controlled, such as intensity of agglutination and cytotoxic action. However, the purpose of the present work, namely, to determine the differences between the serum and leucocytes of resistant and susceptible animals with respect to their opsonic and phagocytic activities is fulfilled by

the conditions of the above experiments. Numerous repetitions of different parts of Experiment 4 gave essentially the same results.

TABLE IV.

*Opsonic Action of Serum of Resistant and Susceptible Animals.*

Sensitized pneumococci + leucocytes + serum diluted 1:5.

Kind and quantity of sensitizing serum		Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
					Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
Resistant animals		cc.				
	Cat 10 cc.	0.1	Cat	Cat	96	91
	" " "	"	Rabbit	Rabbit	56	29
	Sheep 10 cc.	"	Cat	Cat	89	74
	" " "	"	Rabbit	Rabbit	66	44
	Pig 10 cc.	"	Pig	Pig	91	86
	" " "	"	Dog	Dog	53	33
	" " "	"	Rabbit	Rabbit	83	73
Susceptible animals	" " "	"	Guinea pig	Guinea pig	96	88
	Horse 10 cc.	"	Rabbit	Rabbit	27	19*
	Rabbit 10 cc.	"	Rabbit	Rabbit	0	0
	" " "	"	Sheep	Sheep	0	0
	" " "	"	Cat	Cat	0	0
	Guinea pig 10 cc.	"	Guinea pig	Guinea pig	11	0
	" " " "	"	Pig	Pig	5	0
	" " " "	"	Dog	Dog	1	0
Controls with unsensitized pneumococci			Rabbit	Rabbit	0	0
			Cat	Cat	0	0
			Sheep	Sheep	0	0
			Pig	Pig	2	0
			Dog	Dog	1	0
			Guinea pig	Guinea pig	0	0
			Rabbit	Rabbit	0	0

\* Agglutination massive and could be only partially broken up.

A study was made also of the opsonic activity of the blood serum of birds. As this part of the work is less complete than that on mam-

mals, a detailed report is reserved for a future communication. Suffice it to say here that well marked opsonic action was demonstrated with the homologous serum and leucocytes of the chicken and pigeon although not as pronounced as that shown by mammalian blood. Tests with alien blood elements failed, except in one or two instances, to show any degree of phagocytosis.

*Significance of Normal Antipneumococcus Opsonins.*

Experiments were next undertaken with the purpose of ascertaining as far as possible the importance of opsonic action in the pneumococcus-destroying processes of the body. In previous studies on the pneumococcal action of serum-leucocyte mixtures of the dog and the cat it was found that inactivation of the serum at 56°C. deprived the mixture of its pneumococcus-killing action. The further finding in the present work that heating to 56°C. largely destroys the opsonic properties of the normal serum suggests that pneumococcal power of the blood is dependent on the opsonic activity of the serum. To test this presumption a series of experiments was performed with the serum and leucocytes of the dog, cat, sheep and pig, comparing opsonic action with pneumococcal potency. A specimen protocol is shown in Table V.

*Experiment 5.*—(Table V.) Serum and leucocytes obtained from a normal full grown pig. The growth inhibition tests were carried out as detailed in previous papers (23, 28). The opsonic tests were performed as in the preceding experiments. The sedimented organisms from both the inactivated and active serum tubes showed agglutination which was most marked with the fresh serum, least with the 65°C. heated serum. Leucocyte extracts were prepared in two ways: (1) by shaking washed leucocytes suspended in Locke's solution with glass beads for 2 hours, at the end of which time only nuclear remains and stroma were seen; and (2) by grinding leucocytes in very fine sand for 20 minutes, which resulted in their complete maceration.<sup>5</sup> These suspensions were then centrifuged at high speed and the clear supernatant fluid used in the test.

The pronounced pneumococcal action shown by the serum-leucocyte mixtures of all the animals tested was entirely abolished

<sup>5</sup> Leucocyte extracts were prepared as well in the experiments with dog, cat and sheep serum-leucocyte mixtures by freezing and thawing and also extracting for 24 hours with distilled water.

when heated serum was substituted for fresh serum. Comparative opsonic tests on the heated and unheated sera showed that the former

TABLE V.

*Opsonic Activity and Pneumococcal Power of Pig Serum and Leucocytes:  
Action of Leucocyte Extract and Effect of Serum Inactivation.*

*A. Growth Inhibition Test.*

Pig serum 0.3 cc. + pig leucocytes or extract 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension	Growth as shown by color changes* at hrs			Survival of pneumococci at 72 hrs.	
		17	42	72	Stained film	Culture
	cc.					
Fresh serum	0.01	0	0	0	0	0
	0 001	0	0	+	+	+
	0 0001	0	0	0	0	0
	0 00001	0	0	0	0	0
	0 000001	0	0	0	0	0
Serum inactivated at 56°C.	0 0001	+	++++		+	
	0 00001	0	++++		+	
	0 000001	0	++	++++	+	
	0 0000001	0	+++	++++	+	
Serum inactivated at 65°C.	0 0001	++++			+	
	0 00001	++++			+	
	0 000001	++++			+	
	0 0000001	++++			+	
Leucocyte extract (shaken)	0 0001				+	
	0 00001				+	
	0 000001				+	
	0 0000001				+	
Leucocyte extract (ground)	0 0001				+	
	0 00001				+	
	0 000001				+	
	0 0000001				+	
Controls with fresh serum only	0 000001				+	
	0 0000001				+	

\* Degrees of methemoglobin formation.



TABLE V—*Concluded.**B. Opsonic Test.*

Pig serum-sensitized pneumococci + pig leucocytes + pig serum 1:5 dilution.

Quantity of sensitizing serum	State of sensitizing serum	Amount of pneumococcus suspension	Degree of phagocytosis		
			Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs	Per cent of packed cells; leucocytes containing 20 or more pairs
<i>cc.</i>		<i>cc.</i>			
5	Fresh	0.1	93	87	25
"	Inactivated at 56°	"	57	32	3
	C.				
"	Inactivated at 65°	"	35	9	0
	C.				
Control with unsensitized pneumococci. . .			2	0	0

had lost much of their opsonic potency but still retained varying degrees of this property. The opsonins of the pig were found to be the most resistant to heating. As indicated in Table V pig serum heated to 56°C. for half an hour showed well marked opsonic activity, though considerably less than that of the fresh serum. This finding in association with the retardation of growth produced by the 56° heated serum and leucocytes suggests the possibility that while the heated-serum opsonins can still cause phagocytosis, the sensitization produced by opsonins thus injured is inadequate for complete intracellular digestion.<sup>6</sup> Heating the sera of the dog and the cat to 56°C. destroyed their opsonic properties almost completely and there was no growth retardation in the growth inhibition tests with the heated serum and leucocytes of these animals. Sheep serum opsonins were more resistant though not as much so as those of the pig. That part of the experiments dealing with the effect of leucocyte extracts will be taken up later.

<sup>6</sup> That the phagocytosis observed was not due to reactivation of the heated opsonins by the small amount of fresh serum present in the pipette mixture, was determined by comparing the effect of adding to heated serum-sensitized pneumococci and leucocytes, heated serum, fresh serum and no serum. The resulting degree of phagocytosis in all three mixtures was practically identical.

The results of the foregoing experiments, which were repeated many times with substantially the same findings, indicate that opsonins must play a very important, if not decisive, rôle in the pneumococcal action of serum-leucocyte mixtures. In the absence of opsonins the leucocytes of pneumococcus-resistant animals apparently do not possess any greater ability either to engulf or to cause destruction of virulent pneumococci than do the leucocytes of susceptible species. There still remained the possibility, however, that the leucocytes of the former type of animal may possess within their cell substance pneumococcus-destroying principles which are not present in the cells of the latter. Experiments were accordingly devised in an attempt to determine whether such differences could be demonstrated in the leucocytes of the several animal species studied above.

First, observations were made on the rate of intracellular digestion of dog and cat serum-sensitized pneumococci by dog, cat and rabbit leucocytes. Capillary pipette mixtures were examined microscopically after intervals of 1 to 6 hours incubation. The rabbit leucocytes appeared to digest the engulfed pneumococci fully as effectively as those of the dog and cat. More conclusive information as to the ultimate fate of the ingested organisms was sought by adding pneumococci sensitized by dog serum,<sup>7</sup> both in the active and lag phase, to rabbit serum-leucocyte mixtures, which were then tested for growth-inhibitory effect. The rabbit leucocytes failed to show any pneumococcal action. This experiment was repeated with sheep serum from which the anti-rabbit cytotoxins had been removed by successive additions of rabbit blood cells at 5°C. The result was the same. Likewise, rabbits injected with small quantities of dog and cat serum-sensitized pneumococci, both with and without the removal of the sensitizing serum, invariably succumbed to pneumococcus infection. However, the results of these tests are unsatisfactory from the standpoint of determining the pneumococcus-destroying powers of the leucocytes because cytotoxic action of the alien serum cannot be completely eliminated.

The action of leucocyte extracts on pneumococci was then tested, as shown in Table V. Extracts of leucocytes from the dog, cat, sheep and pig were found to be without pneumococcal properties. The explanation of the discrepancy between our results and those of former workers may well be found in the reaction of the extracts used. Rous (29) has recently shown that the protoplasm of the leucocyte is distinctly acid. In our work the use of adjusted and buffered solu-

---

<sup>7</sup> These organisms were mixed with serum in sealed tubes and agitated during incubation so as to secure complete sensitization of all the pneumococci. The serum was completely removed after centrifugation.

tions and the addition of a relatively large quantity of serum to the leucocyte extract probably acted to maintain its reaction at about that of blood. The extracts

TABLE VI.

*Comparative Pneumococcal and Opsonic Activity of Adult Rabbit and Young Hare Serum and Leucocytes.*

*A. Growth Inhibition Test.*

Serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Serum	Leucocytes	Pneumococcus suspension	Growth as shown by color changes at hrs.			Survival of pneumococci at 72 hrs.	
			16	48	72	Stained film	Culture
Adult rabbit	Adult rabbit	cc.					
		0.01	+++	+++++		+	
		0.001	0	++	+++++	+	
		0.0001	0	0	0	0	0
		0.00001	0	0	0	0	0
		0.000001	0	0	0	0	0
	Young hare	0.01	++	+++++		+	
		0.001	0	++	+++++	+	
		0.0001	0	0	0	0	0
		0.00001	0	0	0	0	0
		0.000001	0	0	0	0	0
		0.0000001	0	0	0	0	0
Young hare	Adult rabbit	0.01	+++	+++++		+	
		0.001	+++	+++++		+	
		0.0001	+	+++++		+	
		0.00001	0	+++++		+	
		0.000001	0	+++++		+	
		0.0000001	0	+++++		+	
	Young hare	0.01	+++++			+	
		0.001	+++	+++++		+	
		0.0001	++	+++++		+	
		0.00001	++	+++++		+	
		0.000001	+	+++++		+	
		0.0000001	0	+++++		+	
Controls with serum only							
Adult rabbit .....		0.0000001				+	
Young hare.....		0.0000001				+	

TABLE VI—*Concluded.**B. Opsonic Test.*

Sensitized pneumococci + leucocytes + serum diluted 1:5.

Kind and quantity of sensitizing serum	Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
				Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
Adult rabbit 5 cc.	0.05	Adult rabbit	Adult rabbit	65	48
" " " "	"	Young hare	Young hare	60	47
Young hare 5 cc.	0.05	Adult rabbit	Adult rabbit	0	0
" " " "	"	Young hare	Young hare	4	1
Controls with unsensitized pneumococci		Adult rabbit	Adult rabbit	0	0
		Young hare	Young hare	0	0

employed by previous investigators may have been acid and hence acted as unfavorable media for the viability of pneumococci. Leucocyte extracts prepared with unbuffered neutral solutions were indeed found to have a slightly higher H ion concentration than the original solution used.

Although largely negative in character, the above findings contribute further evidence in support of the probability that the different animal leucocytes behave in a like manner toward pneumococci. Much more direct evidence of the determining influence of opsonic action in intracellular digestion and natural resistance was obtained from a comparative study of the sera and leucocytes of adult and young rabbits or hares. As was shown in a previous communication (24), the relative resistance of full grown rabbits against certain strains of pneumococcus is associated with the presence of pneumococcal properties in their blood which are entirely lacking in the highly susceptible young animal. It was found possible to analyze the nature of this difference in blood characteristics within the species by the use of methods that had failed in attempts with alien serum and leucocytes.

*Experiment 6.*—(Table VI.) Adult rabbit serum and leucocytes obtained from a white male rabbit, weight 1500 gm. The young hare leucocytes were secured from a young Belgian hare, weight 520 gm., and the serum from this same hare and another weighing 490 gm. The growth inhibition test was carried out as before. The organism employed was a Type II pneumococcus, which possessed but little virulence for adult rabbits or hares. 0.1 to 0.01 cc. was required to kill the full grown animal, but 0.000001 cc. killed 500 to 600 gm. Belgian hares in 2 to 3 days. For the phagocytic test, pneumococci in the active growth phase were used and sensitization was carried out for 30 minutes. The test was conducted otherwise as previously. The pneumococci sedimented from the adult rabbit serum showed agglutination, those from the young rabbit serum were unclumped.

The results of the experiment are shown in Table VI. A serum-leucocyte mixture consisting of adult rabbit serum and either adult rabbit or young hare leucocytes was found to possess marked growth-inhibitory and pneumococcus-destroying properties. On the other hand, mixtures composed of young hare serum and adult rabbit or young hare leucocytes were entirely lacking in this action. This difference in the effectiveness of the two kinds of serum is to be accounted for by the finding that the large rabbit serum showed well marked opsonic activity both with its own and the young hare leucocytes, while in the serum of the young hare no opsonins could be demonstrated. In these two animals of widely varying susceptibility to pneumococcus infection it seems evident that resistance is dependent upon the presence of opsonins in the serum, since the leucocytes of the young hare are quite as effective in digesting adult serum-sensitized pneumococci as are the leucocytes of the full grown, highly refractory animal.

It is to be noted that actively growing organisms were used in the opsonic test. With Type II pneumococci in the lag phase, agglutination was so massive and tenacious as to interfere markedly with phagocytosis.

#### *Normal Antipneumococcus Agglutinins.*

As recorded in the preceding protocols, agglutination occurred wherever opsonic action was detected. Experimental conditions found to be optimum for the demonstration of opsonic activity likewise brought out the most pronounced agglutination. Within certain limits the degree of agglutination observed paralleled the extent to

which the sensitized pneumococci were phagocyted. This parallelism ceased when the intensity of the agglutination reached the point where the clumps were so large that they could no longer be broken up. Heating the serum caused a diminution in both its agglutinative and opsonic properties to, roughly, the same degree. That this clumping of the pneumococci sedimented from the serum of pneumococcus-resistant animals was a true agglutination is shown by the fact that the clumps could never be completely comminuted even after prolonged mixing with a fine capillary pipette. While the suspension might have a smooth appearance (it usually retained a finely granular character), agglutinated masses of pneumococci were always observed microscopically except in instances where the macroscopic clumping had been very slight.

It is not improbable that the packing of the organisms by centrifugation at high speed had much to do with the successful demonstration of these normal serum agglutinins. Gates showed that the agglutination of meningococci could be greatly hastened and intensified by centrifuging (30).

#### DISCUSSION.

The results of the foregoing experiments strongly suggest that the differences in resistance which certain mammals normally exhibit toward pneumococcus infection are to be accounted for chiefly by the concentration of antipneumococcus opsonins present in their blood. While the markedly resistant animals possess a relatively high concentration of these immune substances (or serum state), the blood of susceptible ones is so poor in this property that it cannot deal with the highly virulent pneumococci but still has a sufficient concentration to sensitize and thereby make possible the destruction of pneumococci of low virulence for the species.

It is realized that the data presented do not entirely exclude the existence of blood cellular differences between resistant and susceptible animals, but the marked phagocytic and intracellular digestive activity shown by the leucocytes of susceptible animals for pneumococci sensitized by the serum of resistant species, makes it seem probable that no striking variations in this function obtain among the several kinds of leucocytes studied. In support of this

inference is the previous finding that the addition of a small quantity of specific immune serum to the serum-leucocyte mixture of the rabbit confers on it marked lethal power for highly virulent pneumococci (31), thus showing that the leucocytes of a susceptible animal are capable of killing such organisms when adequately sensitized.

The degree and type of pneumococcal action which can be demonstrated in the resistant mammal's blood, together with the cellular picture of the pneumococcus lesion, indicates that in these animals the polymorphonuclear leucocytes are principally responsible for the destruction of pneumococci. Phagocytosis by the fixed tissue cells of the reticulo-endothelial system may, however, play a contributory rôle. The possibility that the body possesses also a mechanism for the extracellular dissolution of pneumococci is by no means excluded even though no conclusive evidence for the existence of such a process has been thus far found.

The general parallelism found to exist between the agglutinative and opsonic properties of the serum of pneumococcus-resistant animals provides some additional evidence in favor of the view held by many that these two reactions are different manifestations of a single immune process or substance.

#### SUMMARY.

A study was made of the pneumococcal action of serum-leucocyte mixtures of pneumococcus-resistant animals with a view to determining whether this property of the blood is to be accounted for by the presence of certain serum constituents or by cellular characteristics which are lacking in the blood of susceptible animals. By means of a method specially developed for this purpose, it was found that, after adequate contact with the serum of pneumococcus-resistant animals, virulent pneumococci were phagocytosed actively not only by the homologous leucocytes but also by the leucocytes of other resistant and susceptible animals. On the other hand, pneumococci exposed to the action of the serum of pneumococcus-susceptible animals were not taken up by the leucocytes of either the resistant or susceptible species. All the resistant animals tested, dog, cat, sheep, pig and horse, showed marked opsonic properties in their blood serum which were not found in the serum of susceptible ones, rabbit, guinea pig

and human. There appeared, however, to be no essential difference in the phagocytic activity of the leucocytes from the various animals.

It was then shown that the pneumococcus-destroying power of serum-leucocyte mixtures was entirely abolished when heated serum was substituted for fresh serum and that such heated serum had lost much of its opsonic potency. Neither the living leucocytes alone nor extracts of the leucocytes were observed to exert any killing action on pneumococci. Further evidence of the controlling influence of opsonic action in the antipneumococcus defence mechanism of the blood, and its importance in natural resistance, was afforded by a study of the opsonin content and leucocytic functions of the blood of full grown and young rabbits as related to their widely varying degrees of pneumococcus susceptibility.

#### BIBLIOGRAPHY.

1. Tchistovitch, N., *Ann. Inst. Pasteur*, 1890, iv, 285.
2. Metschnikoff, E., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 689.
3. Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.
4. Kruse, W., and Pansini, S., *Z. Hyg. u. Infektionskrankh.*, 1892, xi, 337.
5. Neufeld, F., and Händel, L., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1912, iv, 554.
6. Ungermann, E., *Arb. k. Gsndhtsamte*, 1911, xxxvi, 341.
7. Hektoen, L., and Ruediger, G. F., *J. Infect. Dis.*, 1905, ii, 128.
8. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.
9. Dold, H., *Arb. k. Gsndhtsamte*, 1911, xxxvi, 419.
10. Pettersson, A., *Z. Immunitätsforsch., Orig.*, 1910, vii, 693.
11. Schneider, R., *Arch. Hyg.*, 1909, lxx, 40.
12. Hiss, P. H., Jr., *J. Med. Research*, 1908, xix, 323.
13. Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.
14. Hiss, P. H., Jr., *J. Med. Research*, 1908, xix, 323.
15. Zinsser, H., *Infection and resistance*, New York, 3rd edition, 1923, 68.
16. Eastwood, A., *Rep. Pub. Health and Med. Subj., Ministry of Health, No. 22*, 1923, 15.
17. Neufeld, F., and Händel, L., *Arb. k. Gsndhtsamte*, 1910, xxxiv, 166.
18. Cecil, R. L., and Austin, J. H., *J. Exp. Med.*, 1918, xxviii, 19.
19. Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1924, xxxv, 330.
20. Burhans, C. W., and Gerstenberger, H. J., *Am. J. Dis. Child.*, 1924, xxviii, 415.
21. Bull, C. G., and McKee, C. M., *Am. J. Hyg.*, 1921, i, 284.
22. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.



23. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xxxix, 219.
24. Woo, S. T., *J. Exp. Med.*, 1926, xliii, 623.
25. Wright, A. E., and Douglas, S. R., *Proc. Roy. Soc., London*, 1903-04, lxxii, 357.
26. Sia, R. H. P., *J. Exp. Med.*, 1926, xliii, 633.
27. Rosenow, E. C., *J. Infect. Dis.*, 1907, iv, 285.
28. Robertson, O. H., Woo, S. T., and Cheer, S. N., *J. Exp. Med.*, 1924, xl, 487.
29. Rous, P., *J. Exp. Med.*, 1925, xli, 379.
30. Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 63.
31. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xl, 467.

# STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

## VI. THE "REACTIVATION" OF THE BACTERIOLYTIC ACTIVITY OF OXIDIZED PNEUMOCOCCUS EXTRACTS.

By JAMES M. NEILL, Ph.D., AND WILLIAM L. FLEMING.

*(From the Department of Bacteriology and Immunology of Vanderbilt University Medical School, Nashville.)*

(Received for publication, May 6, 1927.)

### INTRODUCTION.

In preceding papers (1-4), the interrelations of the oxidation and reduction products of several bacterial hemotoxins were found to be analogous to the relations known to exist between the corresponding products of hemoglobin. That is, the hemolytically inactive oxidation product of the hemotoxin can be converted to the original active substance by reduction, just as "inactive" ferric hemoglobin is converted to "active" ferrous hemoglobin by the same treatment. These reversible relations between active reduced substances and their inactive oxidation products are of interest not only from immunological aspects, but also from the standpoint of the general physiology of the bacterial cell. For example, the pneumococcus hemotoxin, which on last analysis must be recognized as an integral constituent of the bacterial cell, may be converted from the active reduced state to the inactive oxidized state by biological oxidation, and subsequently the reverse change may be induced by the reducing conditions which are developed by the same biological agents when air is excluded. Although demonstrated in this case with a cellular constituent of unknown physiological importance, the same phenomenon may influence the activity of other cell components and thus be an important factor in the maintenance of the activity of the bacterial cells.

In the present investigation, the principles previously demonstrated (1-4) with hemotoxins are applied to a study of the "reactivation" of the oxidized bacteriolytic agent of *Pneumococcus*. This type of bacteriolytic agent is not related at all to the agents involved in the familiar immunological bacteriolytic system. Although it is not known that the bacteriolytic substance of *Pneumococcus* is antigenic in nature, the present paper is included in the series of studies on the oxidation and reduction of immunological substances because

of the apparent similarity between the reactivation of the bacteriolytic activity and the reactivation of the previously reported hemotoxins.

The bacteriolytic enzymes or agents have been studied chiefly by Emmerich and Löw and their associates (5). These authorities have described "enzymes" of this type from a number of different bacteria, and would include them in the group of nucleases as they are believed to act upon the nucleoprotein of the bacterial cell. Emmerich (6) believed that the autolysis or dissolution of pneumococcus cultures depended upon the action of a bacteriolytic enzyme derived from these bacteria, but did not report an actual demonstration of the enzyme action. The existence of the bacteriolytic enzyme was proved by Avery and Cullen (7) who described it as a thermolabile, intracellular substance which causes lysis of the dead bacterial bodies of pneumococci. Unlike some of the enzymes of this type (the "heteroform" enzymes of Emmerich), the pneumococcus enzyme possesses a considerable degree of specificity, and is without effect upon most other Gram-positive cocci. More closely allied cocci, such as *Streptococcus viridans*, are attacked to some extent, but the action is not nearly so pronounced as upon pneumococci themselves. Emmerich's belief that the bacteriolytic enzyme acts upon the nucleoprotein of the bacteria, may explain the limitations of the apparent species specificity of Avery and Cullen's enzymes. If the nucleoprotein fraction serves as substrate, the slight but definite susceptibility of certain *Streptococcus viridans* to the pneumococcus enzyme may be paralleled by the immunological relations between the nucleoproteins of the same bacteria which have been demonstrated by Lancefield (8). The possible relation of the bacteriolytic enzyme to the rapid autolysis of pneumococci is discussed by Avery and Cullen (7).

To avoid confusion, it is desirable to point out that bacteriolytic agents of this type are distinctly different from the *bacteriolysin* which is a component of the more familiar immune bacteriolytic system. The distinction between them is analogous in many respects to the distinction between the bacterial hemotoxins and the immunological hemolysins (1). The bacteriolytic "enzymes," like the hemotoxins, are products of the bacterial cell, and may be considered as primarily toxic substances, since they possess in themselves the property of causing lysis of the appropriate bacteria. The bacteriolysins, on the other hand, are sensitizing antibodies and unlike the bacteriolytic "enzymes," their lytic action is dependent upon the cooperative mechanism of the sensitizing bacteriolysin and alexin (complement).

It is evident from the foregoing review that the bacteriolytic agent of *Pneumococcus* represents a thermolabile component of the bacterial cell. In previous studies on the oxidation-reduction activities of pneumococci (9), it was shown that the bacteriolytic substance may be inactivated by oxidizing agents formed in the presence of air by

certain other constituents of the same bacterial cells as those from which the "enzyme" itself was derived. The present paper is concerned with the subsequent "reactivation" of the bacteriolytic activity by biological reduction. The question of the true "enzyme" nature of the bacteriolytic agent is of no immediate consequence since this substance is utilized in the present study merely as an example of a cellular constituent which is inactivated by oxidation.

## EXPERIMENTAL.

### *Methods.*

*"Oxidized" and "Unoxidized" Pneumococcus Extracts.*—The pneumococcus extracts consisted of the filtered extract of a concentrated suspension of unwashed pneumococcus cells which had been disrupted by repeated freezings and thawings (10). This type of extract (the "complete system" type) (11) contains easily oxidized substances which upon exposure to air form oxidizing agents which in turn inactivate much of the original bacteriolytic activity of the extract.

The term "oxidized" extract denotes pneumococcus extracts in which at least a part of the bacteriolytic agent has been oxidized by exposure to air in unagitated Erlenmeyer flasks. The degree of inactivation of the bacteriolytic agent under these conditions is determined not only by the time of exposure but also by the oxidizing activity of the bacterial extract. In the previous investigations in which the oxidation was first described (9) the inactivation was not so pronounced as with the more active extracts which have been employed in the present study.

The term "unoxidized" extract, as employed in this paper, refers to pneumococcus extracts which have been protected from oxidation by a heavy seal of vaseline. Under these conditions, the bacteriolytic activity is relatively stable as it has been found to be unimpaired after 3 years storage in the ice box.

*Reduction Treatment Employed in the Reactivation of the Oxidized Extracts.*—The method employed for the reactivation of the bacteriolytic activity of the oxidized extracts was essentially the same as that utilized in the reactivation of the hemotoxins by biological reduction.

Equal portions of the oxidized extract were placed in each of two narrow test-tubes. A measured amount of a suspension of anaerobic bacilli was added to one of the tubes which was then sealed with vaseline. This mixture was incubated at 37°C. for 2 or 3 hours to allow sufficient time for its reduction. An amount of salt solution equal in volume to the suspension of anaerobic bacilli which was added to the first tube, was then added to the second tube of oxidized extract. This mixture, which served as a control of the bacteriolytic activity of the oxidized extract before its reduction treatment, was placed in the ice box until the time of the measurement of the bacteriolytic activity. A third tube containing a similar mixture of broth plus anaerobic bacilli was sealed with vaseline and incu-

bated with the first mixture described. At the end of the period allowed for reduction, the sealed mixture of oxidized extract and anaerobic bacilli and the control mixture of broth plus anaerobic bacilli, were centrifuged at high speed to remove the bacteria. The supernatant fluids of the reduction mixtures which were utilized in the bacteriolytic tests proper were entirely free from the bacilli used as reducing agents.

This method of reduction treatment was chosen as it afforded a means of obtaining reduced fluids for the bacteriolytic tests without the introduction of reducing agents into the final bacteriolytic tests themselves.

*Measurement of Bacteriolytic Activity.*—In most of the experiments, the measurements of the bacteriolytic activity consisted in comparisons of the activities of oxidized extracts, before and after treatment with the biological reducing agent. Heat-killed suspensions of pneumococci (Type II) were used as substrates in the enzyme tests. Equal amounts of the three mixtures described above were added to separate series of tubes containing the heated pneumococci. The bacteriolytic test mixtures were incubated in the water bath, and the degree of bacteriolysis was noted at varying intervals by observing the relative opacity of the tubes and by microscopic examination of stained fields.

It was found advisable to centrifuge the bacteriolytic test mixtures before the final measurements of bacteriolysis. The supernatant fluids were removed and the bacterial sediment was resuspended in 0.5 cc. of sterile salt solution. Comparisons of the relative turbidities of these concentrated suspensions furnished a convenient means of determining differences in the degree of bacteriolysis. These measurements were checked by the microscopic examination of films prepared from the same suspensions.

### *Reactivation of the Oxidized Bacteriolytic Agent by the Reducing Action of Bacteria.*

In the previous studies on the hemotoxins (1-4) it was shown that the inactive oxidation products could, in the absence of air, be converted to the active form by the reducing action of bacteria. Experiments were made to determine if the same biological reducing agents can induce a similar reactivation of the bacteriolytic agent.

Cultures of Anaerobic Bacillus T (a non-hemolytic organism isolated from a wound and morphologically resembling *B. tetani*) were grown in 50 cc. centrifuge tubes under vaseline seal; these cultures were centrifuged and the bacterial cells were suspended in 1.0 cc. of sterile salt solution.

The following test mixtures were prepared:

- (1) 1.0 cc. oxidized pneumococcus extract + 0.5 cc. salt solution.
- (2) 1.0 cc. oxidized pneumococcus extract + 0.5 cc. suspension of Anaerobic Bacillus T.

- (3) 1.0 cc. broth + 0.5 cc. suspension of Anaerobic Bacillus T.  
 (4) 1.0 cc. unoxidized pneumococcus extract + 0.5 cc. salt solution.  
 (5) 1.0 cc. unoxidized pneumococcus extract + 0.5 cc. suspension of Anaerobic Bacillus T.

All four mixtures were centrifuged after a 2 hour period allowed for reduction and the supernatant fluids utilized in the bacteriolytic tests. The further procedure involved in the bacteriolytic tests has been described under "Methods." Mixtures 4 and 5 were prepared in order to prove that the treatment employed for reduction did not increase the bacteriolytic activity unless the extract had previously been oxidized.

TABLE I.

*"Reactivation" of the Oxidized Bacteriolytic Agent by the Reducing Action of Bacteria.*

Amount of pneumococcus extract	Bacteriolytic activity of pneumococcus extract				
	"Oxidized" extract		"Unoxidized" extract		Controls on bacterial reducing agent
	Not treated with bacterial reducing agent	After treatment with bacterial reducing agent	Not treated with bacterial reducing agent	After treatment with bacterial reducing agent	
cc.					
0.12	+	++++	++++	++++	0
0.02	±	++	++	++	0

0 = no lysis.

± = slight lysis, but most of the cells still intact.

+ = distinct lysis; few intact cells possessing Gram-positive staining properties.

++ = marked lysis; no Gram-positive cells and few cells retaining distinct morphology.

+++ = lysis almost complete; no cells possessing distinct morphology.

++++ = complete lysis; film shows nothing but amorphous Gram-negative detritus.

Mixture 3, the control on the bacterial reducing agent, never showed any evidence of bacteriolytic action. The results of the tests of this mixture are presented in the protocol of this experiment under the heading "Controls on bacterial reducing agents." Similar control tests were made in all the following experiments, although it has not seemed necessary to present them in the protocols.

The results of the experiment are given in Table I.

It is evident in Table I that oxidized pneumococcus extract which has lost much of its bacteriolytic activity regains at least most of its original activity after treatment with anaerobic bacilli in the absence

of air. The anaerobic bacilli were removed from the extracts by centrifugation before the bacteriolytic tests so that the supernatant fluid used in the test itself should be free from foreign bacteria. The negative results exhibited in the "control on bacterial reducing agent" prove that the bacilli employed in the previous reduction treatment contributed no substances to cause the disintegration or bacteriolysis of *Pneumococcus*. The fact that the same treatment which reactivated the oxidized extract did not increase the degree of activity of the unoxidized extract is a further control and proves that the reactivation occurs only in extracts which contain inactive oxidation products.

TABLE II.

*Attempt to Reactivate, by Reduction, the Bacteriolytic Activity of Heat-Inactivated Pneumococcus Extracts.*

Treatment of pneumococcus extract	Bacteriolytic activity of pneumococcus extract			
	Heated		Unheated	
	Unoxidized extract	Oxidized extract	Unoxidized extract	Oxidized extract
Before treatment with bacterial reducing agent	0	0	++++	+
After treatment with bacterial reducing agent	0	0	++++	++++

*Attempt to "Reactivate" the Bacteriolytic Activity of Heat-Inactivated Pneumococcus Extract.*

It has been shown in previous papers (1-4) that the heat-inactivated products of the hemotoxins cannot be "reactivated" by the reduction treatment which successfully restored the original activity to the inactive oxidation products. Experiments were made to determine if the same relation holds true in the case of the pneumococcus bacteriolytic agent.

Both unoxidized and oxidized pneumococcus extracts were included in the experiments. The extracts were heated for 10 minutes at 75°C. which is a period known to be slightly in excess of that sufficient to destroy the bacteriolytic activity (7, 9).

The reduction mixtures employed were analogous to those described in the experiments reported in Table I. The tests for bacteriolytic action were made

by the same procedure. The results of the test with amounts of mixture equivalent to 0.2 cc. of the pneumococcus extract are presented in Table II.

The results of these experiments (Table II) were the same as those obtained in similar experiments with the hemotoxins. Reduction treatment which successfully reactivates the bacteriolytic action of oxidized pneumococcus extract, fails entirely to reactivate either unoxidized or oxidized extracts which have previously been inactivated by heat. This fact indicates that the inactive products formed by heat are distinctly different from the inactive products formed by the oxidation processes which take place in aerated pneumococcus extracts. Since different products are formed, the reactions involved in the in-

TABLE III.

*Species Specificity of the Bacteriolytic Agent in Reactivated Pneumococcus Extract.*

Bacteriolytic agent present in	Bacteriolytic action upon		
	Pneumococci	Hemolytic streptococci	Staphylococci
Original, "unoxidized" pneumococcus extract	++++	0	0
Extract "reactivated" by reduction subsequent to oxidation	++++	0	0
Heated controls of both of the above types of pneumococcus extract	0	0	0

activations by heat and by oxidation must also be of different nature. While the inactivation by oxidation appears to be similar in nature to the reversible change of hemoglobin to methemoglobin, the inactivation by heat can be assumed to represent a protein denaturation.

*Species Specificity of the Bacteriolytic Agent in Reactivated Pneumococcus Extracts.*

Avery and Cullen (7) have shown that the pneumococcus bacteriolytic enzyme is species-specific, causing the rapid disintegration of killed pneumococci, but not attacking the cells of other bacterial species. Hence, experiments were designed to determine whether or



not the bacteriolytic agent in reactivated pneumococcus extracts is likewise without effect upon bacterial cells of other species.

The bacteriolytic tests were made upon heat-killed suspensions of pneumococcus, *Streptococcus hæmolyticus*, and *Staphylococcus aureus*. Both unoxidized pneumococcus extract which had never been exposed to air, and oxidized extract which had been reactivated by bacterial reduction, were added to separate tubes containing the suspensions of each of the different species of bacteria.

A control series was included, in which heat-inactivated portions of both reduced and oxidized pneumococcus extracts were tested against each of the different kinds of bacteria.

The protocol of the experiment is condensed in Table III.

The results of this experiment (Table III) show that the bacteriolytic agent present in reactivated pneumococcus extracts possesses the same species specificity as that exhibited by the original bacteriolytic agent contained in the untreated or original bacterial extract. This fact presents further evidence that the reactivated bacteriolytic agent is identical with the original bacteriolytic enzyme since it is difficult to conceive of an extraneous bacteriolytic substance possessing by chance the same biological specificity.

*Separation of the Hemotoxin and the Bacteriolytic Agent, Both of Which Are Present in Pneumococcus Cell Extracts.*

The similarity in the general nature of the results of the previous investigation of the reactivation of the hemotoxin of *Pneumococcus* to the results of the present study of the reactivation of the bacteriolytic enzyme, makes it highly desirable to determine whether or not the hemolytic and bacteriolytic agents are distinct and separate substances. Both the hemolytic and bacteriolytic properties are functions of one or more constituents of the pneumococcus cell, and hence, both properties are exhibited by the pneumococcus cell solutions. In a previous study of the oxidation of pneumococcus enzymes (9), it was found that the bacteriolytic property of the bacterial extract was more resistant both to heat and to oxidation than is the hemotoxin. Although it is possible to obtain an extract in which all the hemolytic activity has been destroyed by heat or by oxidation treatment without a total inactivation of the bacteriolytic property, this distinction between the labilities of the hemolytic and bacteriolytic properties is not conclusive proof that the two properties (bacteriolysis and hemolysis) are functions of distinct and separate cellular substances.

The following experiments were designed to obtain a more satisfactory separation of the agents involved in pneumococcus bacterioly-

sis and in pneumococcus hemolysis. Pneumococcus extracts which exhibited both the hemolytic and bacteriolytic properties were absorbed with red blood cells to remove the hemotoxin or other substances which are responsible for the hemolytic action of the bacterial extract. Tests were then made to determine whether removal of the hemolytically active substances from the extract likewise removed the active substances concerned in the bacteriolytic action.

*Absorption of the Hemotoxin.*—Pneumococcus extract, which contained both the hemotoxin and bacteriolytic agent in the reduced or active condition, was diluted with 10 times its volume of sterile, cold salt solution. Two 10 cc. portions of the diluted extract were placed in an ice bath and held there for 3 hours, to ensure a temperature of 0°C. before the absorption was attempted. Washed blood cells (rabbit) were likewise held in the ice bath for several hours before the experiment proper was begun.

1 cc. of the cold blood cells was then added to one of the tubes of diluted pneumococcus extract. The tube was shaken gently but thoroughly at the time the blood cells were added, and also at 15 minute intervals during the 4 hour period allowed for the "absorption" of the hemotoxin. No blood cells were added to the second tube of diluted pneumococcus extract, which served as the "unabsorbed" control. At the end of the period allowed for the combination of the blood cells and hemotoxin, 5 cc. portions of the "absorbed" mixture and of the "unabsorbed" control were removed and centrifuged. The supernatant fluid was removed from both the "absorbed" and "unabsorbed" test mixtures. "Titrations" of the bacteriolytic and hemolytic activities of these fluids were then made by the previously described methods.

The hemotoxin titrations of the supernatant fluid of the "absorbed" mixture tested the presence or absence of active hemotoxin after treatment with blood cells. In order to prove that the hemotoxin was removed by actual combination with the blood cell and not simply rendered inactive by the "absorption" treatment, the centrifuged cells were washed once with cold salt solution to eliminate the possibility of traces of free lysin in the residual sediment, and the washed cells resuspended in salt solution. The prompt hemolysis of the "absorbed" cells when the suspension was placed at 37°C. was accepted as evidence of previous combination of the erythrocyte and the hemotoxin.

A further control test was included to eliminate the possibility of the lysin having been inactivated by oxidation during the absorption period. This control consisted in tests for the presence of oxidized lysin in the supernatant fluid by including a duplicate series of hemotoxin titrations with fluids treated with hydrosulfite which would reactivate any of the oxidized form which might have been present.

*Temperature Precautions.*—The success of experiments of this type depends upon differences in the effect of low temperature upon the speeds of the reactions

involved in the "combination" and in the "injury" functions of the hemotoxin. Therefore, it is necessary to take the utmost precautions to avoid even slight rises of temperature in the mixture of pneumococcus extract blood cells during the "absorption" period. The apparatus (pipettes, etc.) was chilled in the ice box before use in the experiment and all stages of the manipulation including the centrifugation were carried out at a temperature of 2°C.

The results of this experiment (Table IV) present proof that different substances are involved in the bacteriolytic and hemolytic activities of pneumococcus extracts. The tests of the hemolytic activity of supernatant fluid (Table IV) proved that the hemotoxin was completely removed from the "absorbed" fluid. The prompt hemolysis which took place when the "absorbed" blood cells were resuspended in warm salt solution, proved that the hemotoxin was actually combined with

TABLE IV.

*Separation of the Hemotoxin and the Bacteriolytic Enzyme Contained in Pneumococcus Extracts.*

Pneumococcus extract	Bacteriolytic activity		Hemolytic activity	
	0.20 cc.	0.02 cc.	0.20 cc.	0.02 cc.
After absorption with red blood cells	++++	+	0	0
Unabsorbed	++++	+	++++	++++

the red cells and not simply rendered inactive by the absorption treatment.

Unlike the hemotoxin, the bacteriolytic agent does not combine with red blood cells. The pneumococcus extract from which all the hemotoxin has been removed by "absorption," still exhibits marked bacteriolytic properties. These results are evidence that the hemotoxin and bacteriolytic agent contained in pneumococcus extracts represent two distinct substances, only one of which possesses the property of combining with erythrocytes at low temperature. This proof of the separate identity of the hemotoxin and bacteriolytic agent is not only of considerable importance in itself, but it shows that the reactivation of the bacteriolytic agent represents a new example of the restoration of the original activity of a pneumococcus cell component by biological reduction.

The "absorption" of the hemotoxin is of theoretical interest from an immunological point of view since it illustrates a fundamental likeness in the action of bacterial hemotoxins and of the true toxins. The actual hemolysis by the hemotoxin, like cell injury by true toxins, includes two reactions—(1) combination of the toxic substance with a cell for which it has specific affinity, (2) injury of the cell with which it has combined. The most satisfying proof that two reactions are involved is furnished by the successful removal of the cell-hemotoxin combination from reaction mixtures held at 0°C.; and by the subsequent rapid injury of the cell which occurs in suspensions of the cell-hemotoxin combination as soon as the temperature is raised. The phenomenon of "absorption" depends upon differences in relative speeds of the two reactions, and not upon the complete inhibition of the second reaction for hemolysis takes place even at 0°C. if the mixtures are allowed to stand for longer periods of time.

*The Reversible Inactivation (Oxidation) and Reactivation (Reduction)  
of the Pneumococcus Bacteriolytic Enzyme, by Living  
Pneumococcus Cells.*

It has been shown in preceding experiments that the inactive oxidation products of the bacteriolytic agent can be converted to the original active condition by reduction processes induced by other bacteria. Since it has been proved that the bacteriolytic substance represents an integral constituent of the pneumococcus cell, it is of considerable importance to determine if the living *Pneumococcus* itself can likewise effect the "reactivation" of the inactive oxidation products of this cellular component. *Pneumococci* manifest either oxidizing or reducing powers dependent upon the presence or absence of air, and in the following experiment tests were made to determine whether these bacteria can induce both the oxidation (inactivation) and reduction (reactivation) of the bacteriolytic agent under the proper conditions of oxygen tension.

By using only young unautolyzed pneumococci, it was possible to remove the bacteria by centrifugation and to obtain a supernatant solution which included no bacteriolytic substances introduced by the microorganisms used as the oxidizing and reducing agents. Control mixtures of broth and pneumococci were prepared at the time of each oxidation and reduction treatment to prove this point.

The experiment was similar in nature to previous experiments in which pneumococci were employed in the reversible oxidation and reduction of hemoglobin (12)<sup>1</sup> and of pneumococcus hemotoxin (1).<sup>2</sup> The procedure included the successive reversible oxidation and reduction of the bacteriolytic agent by fresh suspensions of young, living pneumococci. The oxidation was effected by aeration of the mixture of enzyme solution and bacterial cells. After allowing time for the oxi-

<sup>1</sup> Neill (12), Table II.

<sup>2</sup> Neill (1), Table V.

duction of the bacteriolytic agent, the pneumococci were removed by centrifugation, and tests were made of the bacteriolytic activity of the supernatant fluid. The oxidized solution was then again reduced by adding a fresh suspension of young pneumococci and sealing the reduction mixture. At the end of the period allowed for reduction, the mixture was centrifuged and tests were made of the bacteriolytic activity of the supernatant fluid of the reduced solution.

TABLE V.

*The Reversible Inactivation and "Reactivation" of Pneumococcus Bacteriolytic Enzymes by Living Pneumococcus Cells.*

Oxidation or reduction mixture	Treatment	Oxidizing or reducing agent	Period of oxidation or reduction	Bacteriolytic activity	
				(Amounts of mixture in terms of original pneumococcus extract)	
				0.2 cc.	0.02 cc.
Solution I	Original pneumococcus extract	Sterile pneumococcus extract sealed from air	hrs.	++++	+++
Solution II	Solution I after oxidation	Sterile pneumococcus extract exposed to air	24	++	+
Solution III	Solution II after reduction	Young, intact pneumococci in absence of air	5	++++	++
Solution IV	Solution III after oxidation	Young, intact pneumococci in presence of air	2	+	±
Supernatants of controls (broth plus young pneumococci) for each reduction and oxidation mixture				0	0

The sequence of conversions of the bacteriolytic agent in the same bacterial extract from the active reduced form to the inactive oxidized form, and back again, is outlined in Table V. Solution I represents the original (unoxidized) pneumococcus extract at the beginning of the experiment, in which all the bacteriolytic agent is in the active state. The first oxidation was effected by the oxidizing agents formed in the extract itself when exposed to air. It is believed that the successive oxidations (inactivations) and reductions (reactivations) in Solutions

II, III, and IV can be referred directly to the oxidizing or reducing action of the young pneumococci added to the respective mixtures.

The results of this experiment (Table V) indicate that living pneumococcus cells possess the ability either to oxidize (inactivate) or to reduce (reactivate) the bacteriolytic agent. These results are analogous to the results of a similar experiment with the hemotoxin in which it was shown that pneumococci possess a like capacity to oxidize and reduce reversibly this substance. The direction of the action (oxidation or reduction) is, in each instance, determined by the presence or absence of air. Pneumococci, however, require a longer period of time to effect the reduction of either of these substances than is required for their oxidation under the appropriate conditions.

Both the hemotoxin and the bacteriolytic agent are actual components of the pneumococcus cell. Hence, a demonstration of their reactivation by living pneumococci presents evidence that the inactive oxidation products of certain cellular constituents can be converted to their original active state by reduction processes induced by bacterial cells of the sort from which the cellular substance itself was originally derived.

#### DISCUSSION.

The bacteriolytic agent in pneumococcus extracts is inactivated by oxidizing agents. It is shown in this paper that the bacteriolytic activity of oxidized pneumococcus extracts can be restored by treatment with the biological reducing agents which were used in former studies on the "reactivation" of oxidized hemoglobin and oxidized hemotoxins. By employing biological agents (anaerobic bacteria) for the reduction of the previously oxidized extracts it was possible to remove the reducing agent from the test fluids prior to the measurements of bacteriolytic activity. In this respect, the use of the biological reducing agent seemed preferable to the use of soluble chemical reagents which would be carried over with the test fluids and perhaps affect the subsequent bacteriolytic action.

The reactivation of the bacteriolytic activity of the oxidized extracts by means of biological reducing agents appears to be similar in nature to the previously reported reactivation of the oxidation products of hemoglobin and bacterial hemotoxins. With the latter

substances, there seems to be no question that the reactivation represents a reduction; the following experimental evidence indicates that the reactivation of the bacteriolytic agent involves the same type of reaction. First, numerous controls proved that the biological agents used for the reduction did not themselves contribute any foreign soluble substances possessing bacteriolytic activity. Secondly, the reactivation seems to be dependent upon the presence of oxidation products, for no reactivation occurred except in pneumococcus extracts which had previously been oxidized. Thirdly, the species specificity of the bacteriolytic action of reactivated extracts presented biological evidence that the bacteriolytic agent in the reactivated extracts is identical with that in the original extract. With the support of these three lines of evidence, it is reasonable to believe that the described reactivation of the oxidized bacteriolytic agent, like the similar reactivation of oxidized bacterial hemotoxins, is essentially a reduction process which converts an inactive oxidation product to the original active (reduced) form.

Although its possible enzyme nature is of no immediate consequence, it is important to emphasize the fact that the bacteriolytic substance must be considered as an actual constituent of the pneumococcus cell. Whether or not it is itself of any importance in the life of the cell, it represents an example of a cellular component which is inactivated by oxidation and which can subsequently be reactivated by reduction. These relations appear particularly significant in view of the experimental demonstration (Table V) that living pneumococci themselves can bring about either of these two reactions, under the proper conditions of oxygen tension. Thus, from a physiological point of view, the above results present an example of the conversion of the inactive oxidation product of a cellular constituent to its original active state by means of reduction processes induced by bacterial cells of the sort from which the substance was originally derived. It is evident that if similar reversible relations hold for other cell components, phenomena of this nature must play an important part in the maintenance of cellular activity.

## SUMMARY.

The methods previously employed in the study of hemotoxins have been applied in the present investigation to the oxidation and reduction of the bacteriolytic substance of *Pneumococcus*. It is shown that the bacteriolytic agent, previously inactivated by oxidation, can be "reactivated" by treatment with bacterial reducing agents. Evidence is presented that this "reactivation" represents the reduction of inactive, reversible oxidation products to the original active substance.

The bacteriolytic agent is an integral constituent of the pneumococcus cell, which can be separated from the hemotoxin by absorption with red blood cells in the cold.

## BIBLIOGRAPHY.

1. Neill, J. M., *J. Exp. Med.*, 1926, xlv, 199.
2. Neill, J. M., *J. Exp. Med.*, 1926, xlv, 215.
3. Neill, J. M., *J. Exp. Med.*, 1926, xlv, 227.
4. Neill, J. M., and Mallory, T. B., *J. Exp. Med.*, 1926, xlv, 241.
5. Emmerich, R., and Löw, O., *Z. Hyg. u. Infektionskrankh.*, 1901, xxxvi, 9.  
Emmerich, R., Löw, O., and Korschun, A., *Centr. Bakt., I. Abt., Orig.*, 1902, xxxi, 1. Löw, O., and Kozai, T., *Bull. Coll. Agric. Tokio*, v, cited from Fuhrmann, F., *Vorlesungen über Bakterienenzyme*, Jena, 1907, 64, 65.
6. Emmerich, R., *Centr. Bakt., I. Abt., Orig.*, 1902, xxxi, 585.
7. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1923, xxxviii, 199.
8. Lancefield, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 109; *J. Exp. Med.*, 1925, xlii, 397.
9. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 405.
10. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
11. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1925, xli, 285.
12. Neill, J. M., *J. Exp. Med.*, 1925, xli, 299.
13. Neill, J. M., *J. Exp. Med.*, 1925, xli, 535.





# STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

## VII. THE DIFFERENTIATION OF TETANOLYSIN AND TETANOSPASMIN.

By WILLIAM L. FLEMING.

*(From the Department of Bacteriology and Immunology of Vanderbilt University  
Medical School, Nashville.)*

(Received for publication, May 6, 1927.)

### INTRODUCTION.

It is well known that the tetanus bacillus produces both a hemotoxin and a true, killing toxin ("tetanolysin" and "tetanospasmin"). Historically, the existence of the true toxin was proved (1) about 10 years before recognition of the hemotoxin by Ehrlich in 1898 (2, 3). The properties of tetanospasmin are described in detail in the larger handbooks of immunology, and the literature on the properties of tetanolysin has been reviewed in a previous paper in this series (4). Both of these products of the tetanus bacillus belong to the group of antitoxinogens and possess two fundamental properties in common: (1) they are primarily toxic substances which possess an affinity for particular cells (tetanolysin for erythrocytes and tetanospasmin for nerve cells, especially those of the gray matter of the brain); (2) they are antigenic, invoking the production of a specific neutralizing antibody (antihemotoxin and antitoxin) when systematically injected into an appropriate animal.

Tetanolysin and tetanospasmin have been used as representative "antitoxinogens" in classical experiments, the results of which have been used in the establishment of many of the fundamental principles of immunology. The theoretical importance of these principles, as well as the practical importance of tetanus itself, makes it desirable to obtain a definite differentiation of these two antigenic products of the tetanus bacillus. Although it is generally recognized that they are

distinct substances, there is little experimental evidence available in the literature to show the distinction between them. The most satisfactory differentiation is found in a paper by Madsen (3) (written in Ehrlich's laboratory), who cites, from apparently unpublished experiments of Ehrlich, the following points of difference: (1) the lysin and toxin appear in different proportions in cultures of different strains; that is, some strains are good lysin producers and poor toxin producers and *vice versa*; (2) the lysin is much more labile than the toxin both to "spontaneous deterioration" and to heat; (3) they have different binding relations; when red blood cells are added to tetanus culture fluids the lysin combines with red blood cells while the true toxin or tetanospasmin remains in solution; (4) the lysin and the toxin have separate or specific antitoxins. None of Ehrlich's experimental data is given in support of these observations. (The reference quoted in all the large German source books or handbooks for this differentiation is a short statement by Ehrlich (2) of the discovery of tetanolysin, which mentions only the fourth of the above points of distinction.)

The present paper deals with the differentiation of tetanolysin and tetanospasmin. Both of these substances are antitoxinogens and both are contained in culture fluids of tetanus bacilli. Since most of the differences in properties proposed for their differentiation are quantitative and by no means absolute, the absence of any experimental data in the literature makes it desirable to show the degree of separation of the two substances obtainable on the basis of previously reported (2,3) properties as well as to present new and additional points of distinction.

This report, therefore, includes the results of experiments showing differences in the following properties of these two antigenic products of the tetanus bacillus: (1) time of liberation in broth cultures; (2) effect of absorption with red blood cells; (3) heat lability; (4) susceptibility to oxidation by air.

#### EXPERIMENTAL.

*Methods.*—The tetanolysin and tetanospasmin used in these experiments were contained in broth culture fluids freed from bacteria by filtration through Berkefeld candles. All cultures before filtration were centrifuged under vaseline seal at high speed to remove most of the bacteria and thus insure rapid filtration.

The lysin "titrations" were carried out by the methods described in a previous paper (4) in this series. In some of the experiments, titrations were made with fluids treated with  $\text{Na}_2\text{S}_2\text{O}_4$  so that the measurements would include the hemolytically inactive but reversible oxidation products of the lysin in the culture fluids under examination.

The toxin "titrations" were made by subcutaneous injection of different dilutions of the culture fluids in order to determine the minimum dose causing death and also the minimum dose causing definite paralysis. White mice of approximately the same weight were used; all injections were made at the base of the tail. In spite of the frequent reports of the unreliability of mice for toxin measurements, our results were always regular when mice of uniform age and weight were selected for the experiments.

### *The Effect of Berkefeld Filtration upon Tetanolysin and Tetanospasmin.*

The literature (5) is practically unanimous in stating that tetanus culture fluids cannot be filtered without great losses in the content of both the lysin and the toxin. The loss is commonly ascribed to absorption or combination of the lysin and toxin to the constituents of the filter. Zunz (6) included kaolin, diatomaceous earth, talc, and clay in a study of the absorption of tetanus lysin and toxin. Although he found that absorption of tetanus fluids with some of these substances removed different proportions of the lysin and toxin, none of his absorptions removed all of either the lysin or toxin without also removing a large proportion of the other.

While it did not seem probable that Berkefeld filtration would effect any specific separation of the lysin and toxin, it was desired to use the sterile, filtered fluids for our experiments if the filtration did not cause too great a decrease in the active substances. Hence, comparisons were made of the lysin and toxin contents of a number of different tetanus fluids before and after Berkefeld filtration. A protocol of a typical experiment is given in Table I.

The results in Table I present a comparison of the lysin and toxin contents of the centrifuged supernatant of a 7 day old glucose broth culture, before and after Berkefeld filtration. In the case of the lysin, the slight differences in the hemolytic activity of the filtered and unfiltered fluids are detectable only in the two smallest increments of fluid tested. In the case of the toxin, a slight diminution is indicated by the smaller amount of the unfiltered fluid which was able to cause paralysis. However, with the test increments employed in the titration, no difference could be shown in the minimum lethal dose. Thus, although some loss in both lysin and toxin did occur incident to the filtration, the loss was small and not of the order of magnitude reported in the literature. Similar comparisons made with the fluids of 20 day and 30 day cultures showed no greater losses in either toxin or lysin. (Since no disinfectants were added to the supernatant fluids of the tetanus cultures, it is obvious that they contained a few tetanus spores. It is interesting to note that although a few live spores were

necessarily injected in the tests, their presence in the unfiltered fluids apparently did not affect the toxin titrations.)

It is evident that the loss of lysin and toxin in our experiments was extremely slight in comparison with the great losses attributed to filtration in the literature. Our precautions to prevent loss of the active substances consisted chiefly in attempts to carry out the filtration as rapidly as possible and at a low temperature. (Since pH is frequently an important factor in determining successful filtration, it is desirable to state that the reaction of the fluids at time of filtra-

TABLE I.

*Effect of Berkefeld Filtration upon the Lysin and Toxin Contained in the Centrifuged Supernatants of Tetanus Culture Fluids.*

Lysin titration*			Toxin titration**		
Amount of culture fluid	Hemolytic activity		Amount of culture fluid	Time of death or condition of mice 4 days after injection	
	Before filtration	After filtration		Before filtration	After filtration
cc.			cc.		
0.005	++++	++++	0.003	1 to 2	1 to 2
0.003	+++	+++	0.002	1 to 2	1 to 2
0.002	++	++	0.001	PP	0
0.001	++	+	0.0008	P	0
0.0005	+	±	0.0006	P	0

\* +++++ = complete hemolysis.

+++ = hemolysis approximately 3/4 complete.

++ = hemolysis approximately 1/2 complete.

+

= hemolysis approximately 1/4 complete.

± = hemolysis approximately 1/20 complete.

0 = no hemolysis.

\*\* Numerals indicate time of death in days.

PP = marked paralysis within 4 days.

P = slight paralysis within 4 days.

0 = no paralysis.

tion was approximately pH 6.7 before exposure to air.) The culture fluids were chilled and then centrifuged at high speed, under vaseline seal. The clear supernatant fluid was again chilled to about 5°C. and finally filtered through a dry (V type) Berkefeld filter into a flask packed in ice; the filtrate was sealed with vaseline immediately after filtration. The loss in filtration under these conditions was so slight that it seemed unnecessary to carry out the process in a nitrogen atmosphere as previously employed in the filtration of pneumococcus extracts (7).

*Comparison of the Time of Liberation of the Lysin and Toxin in Tetanus Culture Fluids.*

Both the lysin and the toxin of the tetanus bacillus are commonly considered as "exocellular" bacterial products, since both of them are liberated into the culture fluid and are obtained in filtrates devoid of the bacterial cells. Although both of them are exocellular substances, it seemed desirable to determine whether or not the two products might not be differentiated by the stage of growth during which they are liberated into the culture fluid. The experiments consisted of titrations of the lysin and toxin on the filtrates of cultures of different ages. A condensed protocol of a typical experiment is presented in Table II.

TABLE II.

*Time of Liberation of Lysin and Toxin in Tetanus Culture Fluids.*

Lysin titration				Toxin titration			
Amount of culture fluid	Hemolytic activity			Amount of culture fluid	Time of death or condition of mice 4 days after injection		
	19 hr. culture filtrate	7 day culture filtrate	30 day culture filtrate		19 hr. culture filtrate	7 day culture filtrate	30 day culture filtrate
cc.				cc.			
0.01	++++	++++	++++	0.02	3	1	
0.008	++++	++++	++++	0.01	4 to 5	1	
0.005	++++	++++	+++	0.005	PP	1 to 2	1 to 2
0.003	+++	+++	++	0.004	P	1 to 2	1 to 2
0.002	++	++	+	0.003	P	1 to 2	1 to 2
0.001	+	+	±	0.002	0	1 to 2	1 to 2
0.0005	0	0	0	0.001	0	0	0

The results (Table II) show that tetanolysin and tetanospasmin may be differentiated to a certain extent by the time of their liberation into the culture fluid. The 19 hour culture, with the relatively large inoculum used in this experiment, had already attained its maximum growth, while the 7 day and 30 day cultures must be considered to represent cultures which had passed far beyond the period of active bacterial growth. It is evident from the titrations of the filtrates of these cultures, that the lysin had reached its maximum in the 19 hour culture, while the toxin attained its maximum only in the older cultures. Hence, tetanolysin can be considered as a substance which is elaborated and liberated into the culture fluid during the period of active growth of tetanus bacilli; tetanospasmin, on the other hand, is either elabo-

rated much more slowly or is retained longer within the bacterial cell for it is not set free in the culture fluid until long after the period of bacterial multiplication. From a physiological point of view, it is interesting to observe that these two antigenic products of tetanus bacilli (lysin and toxin) differ from each other in respect to the time of their elaboration or liberation, in much the same way as do the two principal end-products of bacterial nitrogen metabolism; *i.e.*, the lysin, like ammonia, accumulates in the culture fluid during the period of bacterial multiplication; the toxin, like amino acids, accumulates

TABLE III.

*Separation of Tetanolysin and Tetanospasmin by Absorbing Tetanus Culture Fluids with Red Blood Cells.*

Lysin titration			Toxin titration		
Amount of culture fluid	Hemolytic activity		Amount of culture fluid	Time of death or condition of mice 4 days after injection	
	Absorbed with red blood cells	Unabsorbed control		Absorbed with red blood cells	Unabsorbed control
cc.			cc.		
0.06	±	++++	0.0010	1 to 2	1 to 2
0.03	0	++++	0.0007	1 to 2	2 to 3
0.010	0	+	0.0005	2 to 3	2 to 3
0.005	0	±	0.0002	3 to 4	3 to 4
0.003	0	0	0.0001	PP	PP

during later periods when growth has ceased and when the bacterial enzymes alone remain operative.

*Separation of Tetanolysin and Tetanospasmin by Absorbing Tetanus Culture Fluids with Red Blood Cells.*

The technique employed in the absorption of tetanolysin was essentially the same as that utilized in the preceding paper (8) for the absorption of pneumococcus hemotoxin. The same temperature precautions were observed to reduce the possibilities for hemolysis during the absorption. The red blood cells used in the absorption were washed with especial care in order to avoid any possibility of combination of the toxin with lipid or other serum constituents.

The filtrate of a 7 day culture (which contained both the lysin and the toxin) was diluted with 33 volumes of sterile salt solution. The diluted fluid was divided into two portions and put into separate tubes; 1 cc. of washed red blood

cells (rabbit) was added to one tube; no blood cells were added to the second tube, which served as the "unabsorbed control." After a 12 hour period allowed for combination of the lysin with the blood cells, both tubes were centrifuged, the supernatant fluids pipetted off and titrated for lysin and toxin. A condensed protocol of a typical experiment is presented in Table III.

The results of experiments of this type (Table III) furnish the most satisfactory proof that tetanolysin and tetanospasmin are two distinct and separate substances. Although the culture fluid contains both the lysin and the toxin, the lysin can be completely removed by absorption with red blood cells without diminishing the toxin content at all. (The very slight amount of lysin (Table III) left in the absorbed fluid is probably due to the equilibrium established between the erythrocyte-lysin combination and the free lysin. Even this small amount of residual lysin can be removed by a second absorption without decreasing the toxin content.) That the lysin had been removed by true combination with the erythrocytes was proved by the prompt hemolysis of the "absorbed" cells as soon as they were resuspended in warm salt solution; the "absorbed" cells could be washed several times at a low temperature without removing the combined lysin.

The results of this experiment which confirm Ehrlich's observation (3) furnish an interesting example of the specificity of bacterial "antitoxinogens" for particular cells: both of these primarily toxic antigenic products are produced by the same bacillus, but one of them, tetanolysin, possesses a specific affinity for red blood cells, while the other, tetanospasmin, possesses a like affinity for the cells of nerve tissue.

#### *Comparison of the Heat Labilities of Tetanus Lysin and Toxin.*

One of Ehrlich's points of differentiation between tetanus lysin and toxin is their difference in heat lability. Our experiments to determine the difference in the degree of heat lability of the two substances included the following heating treatments: 10 minutes at 55°C.; 5 minutes at 60°C.; and 10 minutes at 60°C. The fluids were protected from air during the heating tests. The results are presented in Table IV.

The results (Table IV) as a whole show that the distinction between the two substances on the basis of differences in heat lability is only quantitative, since it proved impossible to destroy all the lysin without



at the same time inactivating a considerable portion of the toxin. Indeed, when the heating tests were made at 55°C. the toxin inactivation was not much less than the lysin inactivation. While the rates of the inactivation of the two substances seemed approximately the same at 55°C., an increase of 5°C. apparently accelerates the lysin inactivation more than it does the toxin inactivation; and at 60°C. the distinction between the heat labilities of the two substances became more pronounced. 5 minutes exposure of the fluid to 60°C. destroyed practically all the lysin, while it only reduced the toxin to about one-

TABLE IV.

*Comparison of the Heat Labilities of Tetanus Lysin and Toxin.*

Lysin titration					Toxin titration				
Amount of culture fluid	Hemolytic activity				Amount of culture fluid	Time of death or condition of mice 96 hrs. after injection			
	Unheated	10 min. at 55°C.	5 min. at 60°C.	10 min. at 60°C.		Un-heated	10 min. at 55°C.	5 min. at 60°C.	10 min. at 60°C.
cc.					cc.				
0.5	++++	++++	±	0	0.02		1	1 to 2	2 to 3
0.2	++++	++++	0	0	0.01		1 to 2	2 to 3	3 to 4
0.1	++++	++++	0	0	0.006		1 to 2	3 to 4	4 to 5
0.05	++++	++++	0	0	0.003	1 to 2	1 to 2	PP	PP
0.03	++++	++++	0	0	0.002	1 to 2	2 to 3	P	0
0.02	++++	+++	0	0	0.001	1 to 2	3 to 4	0	0
0.01	++++	+++	0	0	0.0007	2 to 3	PP	0	0
0.002	++	0	0	0	0.0005	2 to 3	PP	0	0
0.001	+	0	0	0	0.0002	PP	P	0	0

tenth its original potency. A phenomenon frequently observed in the heat inactivation of toxins is evidenced in the results of the heating at 60°C.: while the greater part of the toxin was inactivated by 5 minutes exposure to 60°C., exposure for twice this period caused no further increase in the M.L.D. of the toxin and only a slight increase in the time of death.

Hence, the results of this experiment show that a more satisfactory distinction between tetanolysin and tetanospasmin can be obtained at 60°C. than at lower temperatures; but that any distinction made between them from the standpoint of heat lability must be quantitative in nature.

*Differences in the Susceptibilities of Tetanolysin and Tetanospasmin to Oxidation by Air.*

Ehrlich's differentiation of tetanolysin and tetanospasmin includes the statement that the "spontaneous deterioration" of the lysin is more rapid than that of the toxin. The following experiment was made to furnish data showing the differences in the susceptibility of the two substances to oxidation when filtrates containing both of them are exposed to air. In these experiments, the lysin titrations included measurements of fluids treated with  $\text{Na}_2\text{S}_2\text{O}_4$  which serves to convert any hemolytically inactive reversible oxidation products of the lysin

TABLE V.

*Differences in the Susceptibilities of Tetanolysin and Tetanospasmin to Oxidation by Air*

Lysin titration					Toxin titration		
Amount of culture fluid	Hemolytic activity				Amount of culture fluid	Time of death or condition of mice 96 hrs after injection	
	Not exposed to air		Exposed to air			Not exposed to air	Exposed to air
	Not treated with $\text{Na}_2\text{S}_2\text{O}_4$	Treated with $\text{Na}_2\text{S}_2\text{O}_4$	Not treated with $\text{Na}_2\text{S}_2\text{O}_4$	Treated with $\text{Na}_2\text{S}_2\text{O}_4$			
cc					cc		
0 05	++++	++++	++	++++	0 004	3 to 4	3 to 4
0 03	++++	++++	+	++++	0 003	PP	PP
0 016	++++	++++	0	++++	0 0024	PP	PP
0 008	++++	++++	0	+++	0 0016	PP	PP
0 004	++	+	0	+	0 0008	0	0

to the active form. These measurements furnished a means of determining whether the loss in hemolytic activity previously termed "spontaneous deterioration" was in fact an oxidation, since only oxidation products could be "reactivated" by the reducing agent.

Tetanus culture filtrate containing both the lysin and the toxin was divided into two portions. One portion was placed in a long narrow tube and sealed with vaseline, the other portion was freely exposed to air in a shallow layer in an Erlenmeyer flask. Both the sealed and the exposed fluids were kept at  $35^\circ\text{C}$  for 24 hours, and then titrated for lysin and toxin by the methods used in the previous experiments. The results are presented in Table V.

The results of this experiment (Table V) show a definite difference in the susceptibility of tetanolysin and tetanospasmin to inactivation by oxidation with air. The exposure to air caused a much greater diminution of the content of active lysin than occurred with the toxin. That the marked difference between the hemolytic activities of the sealed and aerated fluids was due to an actual oxidation was proved by the fact that when the aerated fluid was treated with a reducing agent it regained an activity approximately identical to that of the unexposed fluid. Since only oxidation products of the lysin could be reactivated by the reducing agent, this evidence contributes information not furnished in the literature where the loss in activity is indefinitely referred to as "spontaneous deterioration." In contrast to the marked decrease in active lysin, exposure of the same fluid to air caused no significant loss in the active killing toxin; in fact no loss at all could be detected with the test doses employed in the experiment.

Comparison of the above differences in the ease of oxidation of the lysin and toxin with the previous results on differences in heat lability (Table IV) indicates that one can obtain a more satisfactory separation of these two products by taking advantage of differences in their susceptibility to oxidation than can be obtained by differences in their heat labilities. However, it must not be deduced from the results in Table V that tetanospasmin is not affected at all by exposure to air for we have frequently observed marked losses in tetanus toxin content of fluids which have been exposed to air for several days (9).

#### DISCUSSION.

The preceding experiments have shown the following points of differentiation between tetanolysin and tetanospasmin: (1) the lysin is set free during the period of active growth of the culture, while most of the toxin liberation occurs after bacterial multiplication has ceased; (2) the lysin possesses specific combining affinity toward red blood cells at 0°C., and the toxin does not possess this property at all; (3) the lysin is more heat-labile than the toxin, but the difference in this property is only relative; (4) the lysin is also more susceptible to oxidative inactivation than is the toxin.

Although much of this information has been reported as statements of fact in the literature, an extensive search has revealed no experi-

mental data for the differentiation of tetanolysin and tetanospasmin. The importance of the two antitoxinogens of the tetanus bacillus makes it desirable to present evidence showing the degree of separation of the two products (lysin and toxin) which can be made upon the basis of differences in the above properties. The most satisfactory separation of the lysin and toxin is obtained by absorption with red blood cells which specifically removes tetanolysin without decreasing the tetanospasmin content of the culture fluid. The separation effected on the basis of the other properties is only a relative one. Heating tetanus culture filtrate at 60°C. for short periods, destroys practically all the lysin, but a significant amount of the toxin is also inactivated by the same treatment. The separation of the two substances by means of differences in their susceptibility to oxidation is likewise only a quantitative separation; but if the oxidation treatment is properly chosen, most of the lysin can be inactivated without causing significant losses in toxin.

The separations of the lysin and the toxin by absorption with red blood cells, or by fractional inactivation by heat, or by oxidation yield a fluid containing an increased proportion of the toxin and a decreased proportion of the lysin. The opposite result (*i.e.* a culture fluid containing the maximum amount of tetanus lysin and the minimum amount of tetanospasmin) can be obtained by taking advantage of differences in the period of growth in which the two products are liberated. Tetanus culture fluids if filtered shortly after the end of the period of active bacterial cell growth, contain the maximum amount of lysin, and only minimum amounts of the true toxin; due to the delayed liberation of toxin and the cessation of lysin elaboration, older cultures contain an increased proportion of the true toxin.

The differentiation of the hemotoxin (tetanolysin) and the true toxin (tetanospasmin) of the tetanus bacillus is of particular importance from an immunological point of view, since both of them are antitoxinogens which have been utilized in the illustration of important fundamental principles. While it has become increasingly more evident that bacterial cells and bacterial filtrates represent complex systems containing a number of different antigenic constituents, only a few of these antigens would be included in the antitoxinogen group since the majority of them give rise to sensitizing antibodies rather than the

neutralizing type of antibody. From this aspect, the recognition of tetanolysin and tetanospasmin as distinct and separate substances acquires interest as an example of two primarily and specifically toxic antigens (antitoxinogens) which are produced and liberated in the same culture fluid by one species of bacteria. There are few, if any, other known examples of the production by the same bacteria of two distinct and different antigens of the toxin type in which the true antigenic nature of each of the products is as definitely established as in the case of the hemotoxin and true toxin of the tetanus bacillus.

#### SUMMARY.

Tetanolysin and tetanospasmin possess in common the immunological properties of the group of "antitoxinogens." Both of them are contained in undeteriorated culture fluids of the tetanus bacillus, but each of them represents a distinct and separate antigenic substance. Experimental data illustrating points of difference in their properties are presented in this paper.

#### BIBLIOGRAPHY.

1. Faber, K., *Berl. klin. Woch.*, 1890, xxvii, 717.
2. Ehrlich, P., *Berl. klin. Woch.*, 1898, xxxv, 273.
3. Madsen, T., *Z. Hyg. u. Infektionskrankh.*, 1899, xxxii, 214.
4. Neill, J. M., *J. Exp. Med.*, 1926, xlv, 227.
5. von Lingelsheim, in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1912, iv, 769. von Eisler, M., and Pribram, E., in Kraus, R., and Levaditi, C., *Handbuch der Technik und Methodik der Immunitätsforschung*, Jena, 1908, i, 111. Pribram, E., and Russ, V. K., in Kraus, R., and Levaditi, C., *Handbuch der Technik und Methodik der Immunitätsforschung*, Jena, 1908, i, 208.
6. Zunz, E., *Z. Immunitätsforsch., Orig.*, 1913, xix, 326.
7. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
8. Neill, J. M., and Fleming, W. L., *J. Exp. Med.*, 1927, xlvi, 263.
9. Neill, J. M., and Fleming, W. L., unpublished experiments.

## THE EFFECT OF HEAT ON ANTIBODIES.

By F. S. JONES, V.M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

(Received for publication, May 4, 1927.)

Although it is well known that at certain temperatures antibodies are destroyed or inactivated, nevertheless with few exceptions detailed experiments covering this point are not readily available. T. Smith and Reagh (1) showed that there were two well defined types of agglutinin for the hog cholera bacillus; one they termed flagellar agglutinin, the other, body agglutinin. Beyer and Reagh (2) were able by a series of experiments to differentiate the flagellar and somatic (body) agglutinins by means of heat; the former was unimpaired when heated at 70°C. for 20 minutes, while the action of the somatic agglutinin was markedly impaired under these conditions. Joos (3) had previously called attention to the fact that when typhoid agglutinin was heated at 60–62°C. for 1 hour, a portion of the agglutinin was destroyed. More recently Orcutt (4) took up the study and showed that a temperature of 70°C. destroyed the somatic agglutinin to a considerable extent and 75°C. rendered it completely inactive. On the other hand, 70°C. failed to affect appreciably the flagellar agglutinin and 75°C. rendered it a little less active. It seems definite that certain types of agglutinin react differently to varying temperatures. The data in regard to the behavior of other types of agglutinins, precipitins, and hemolysins are not so definite.

In addition to a study of the specific effect of the various substances upon their respective antigens, it seemed of further interest to ascertain whether antibody was still capable of combining with its antigen although remaining in insufficient quantities to give visible reactions. It might also be possible that heat so affects some of the serum proteins that they no longer respond in a characteristic manner, thus rendering inoperative the usual physical phenomena used to interpret the results.

Complement fixation seemed to meet these objections. With these points in view, it was decided to study the effect of various temperatures on certain antibodies contained in the blood serum of rabbits.

## EXPERIMENTAL.

Rabbits were immunized to various substances and when a sufficiently high titered serum was obtained the animals were bled and the serum collected and stored in the refrigerator. The serum containing agglutinin and hemolysin was

TABLE I.

*The Effect of Various Temperatures on Flagellar Agglutinin.*

	Dilutions of serum										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Un-heated	C*	C	C	C	C	C	+++	+++	+	+-	+-
Heated at °C.											
65	C	C	C	C	C	++++	++	+	+	+-	-
70	C	C	C	C	+++	++	+	+	+	+-	-
75	+++	+++	++++	++++	+++	++	+	+	+-	-	-
80	+	+	+	+	+	+-	+-	-	-	-	-
85	+-	+-	+-	-	-	-	-	-	-	-	-
90	+-	+-	+-	-	-	-	-	-	-	-	-

\* C indicates complete clumping of the antigen; + + + +, marked agglutination without complete clumping of the test fluid; + + +, well defined agglutination; + +, less well defined; +, definitely positive; + -, small deposits of clumped bacilli on the bottom of the tube.

diluted in four parts of normal NaCl solution and heated at various temperatures. The precipitin was diluted with equal parts of NaCl solution and then heated.

In all experiments corrected thermometers were used and the various materials heated in a deep water bath in tightly stoppered tubes for 20 minutes. The tests were always made with the same lot of specific antigen. When complement was employed it was of the same lot and of uniform concentration. The appended protocols afford examples of various observations.

*Experiment 1.*—Agglutinin was prepared by immunizing a rabbit with a motile strain of the hog cholera bacillus. The serum was diluted with four parts of normal

salt solution and distributed in sterile tubes. One part was left unheated, the others were heated for 20 minutes at various temperatures. A portion of the contents of each tube was then tested for "flagellar" agglutinin with the motile strain. Another portion was tested with a non-motile strain of the hog cholera bacillus and a portion of the remainder used in the complement fixation tests further to confirm the findings. The protocols are submitted in Tables I and II.

These experiments substantiate the results of the previous workers. 75°C. for 20 minutes is a critical temperature at which the two agglutinins may be readily separated. The flagellar type resists temperatures considerably higher; even after exposure to 80°C. well de-

TABLE II.

*The Effect of Heat on the Agglutinin for the Non-Motile Strain of the Hog Cholera Bacillus.*

	Dilutions of serum								
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Unheated	C	C	C	C	+++	++	++	+	-
Heated for 20 min. at °C.									
65	+	+	+	+	+	+	+	-	-
70	+	+	+	+	+	+-	-	-	-
75	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-

fined agglutination occurs when the heated serum and antigen are mixed. At temperatures still higher it is possible to show that agglutinin still persists. It will be noted in Table I that in the lower dilutions the reaction has been interpreted as +-. If the small deposits found on the bottom and sides of the tube are examined microscopically it is found that they are composed of clumped masses of bacilli. If this experiment is repeated as a microscopic agglutination test, comparable results are obtained. The bacilli lose their motility and form small, loose clumps.

It might be argued that the antibody still remained in the serum, particularly in the case of the somatic agglutinin, but through some physical or other change in the globulin was incapable of reacting



with its antigen in the characteristic manner. As an additional control, to 10 volumes of the heated antiserum, 1 volume of fresh normal rabbit

TABLE III.

*The Persistence of Antibody in Flagellar Agglutinin Exposed to Various Temperatures.*

Treatment of agglutinin	Antigen	Complement	Antibody	Amboceptor	Hemolysis
Unheated	+	+	+	+	0*
	+	0	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	+-
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	+
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 85°C. for 20 min.	+	+	+	+	+++
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 90°C. for 20 min.	+	+	+	+	+++
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C

\* 0 indicates no hemolysis; C, complete; the plus signs, gradations from a very strong reaction (++++) to barely perceptible hemolysis (+-).

serum was added and the mixture tested. The addition of fresh normal rabbit serum failed to activate the inactivated antibody.

The resistance of the flagellar agglutinin to heat seemed so remarkable that a further control procedure seemed desirable. If it were

TABLE IV.  
*The Effect of Heat on Cow Serum Precipitin.*

	Dilutions of antigen								
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
Unheated	+++*	+++	+++	+++	+++	+++	++	+	+-
Heated for 20 min. at									
°C.									
60	+++	+++	++	++	++	++	+	+	+-
65	++	+	+	+	+	+	+	+-	-
70	+-	+-	+-	+-	+-	+-	+-	+-	-
75	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-

\* Precipitation has been recorded as follows: + + +, the maximum; ++, less; +, weaker but well precipitated; + -, a trace of deposit.

TABLE V.  
*The Effect of Heat on the Complement-Binding Properties of Precipitin.*

Treatment of precipitin	Antigen	Complement	Precipitin	Amboceptor	Hemolysis
Unheated	+	+	+	+	0
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	++++
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	C
	+	-	+	+	0
	+	+	-	+	C
	-	+	+	+	C



possible to show that sufficient antibody remained in heated serum to inhibit complement, the experiment would be further substantiated. With this in view, the whole series was tested, but as the sample exposed to 65°C. behaved like the unheated mixture the results are not given. The antigen consisted of actively motile hog cholera bacilli in normal NaCl solution. The complement was fresh guinea pig serum. The washed red cells of the sheep and the specific amboceptor were employed. These substances were always used in the same concentrations. The antiserum consisted of 0.02 cc. of the serum diluted 1:4 with salt solution. The results are given in Table III.

TABLE VII.  
*The Effect of Heat on Red Cell Agglutinin.*

	Dilutions of agglutinin					
	1:10	1:20	1:40	1:80	1:160	1:320
Unheated	+++*	+++	+++	+++	++	—
Heated at						
°C.						
65	+++	+++	+++	+++	M+	—
70	+++	+++	+	M—	M—	M—
75	++	++	M—	M—	M—	M—
80	M—	M—	—	—	—	—
85	M—	M—	—	—	—	—

\* Agglutination in this table has been recorded as follows: + + +, maximum; + +, less strong; +, clumps definite to the naked eye; M +, the presence of microscopic clumps; M —, the absence of microscopic clumps.

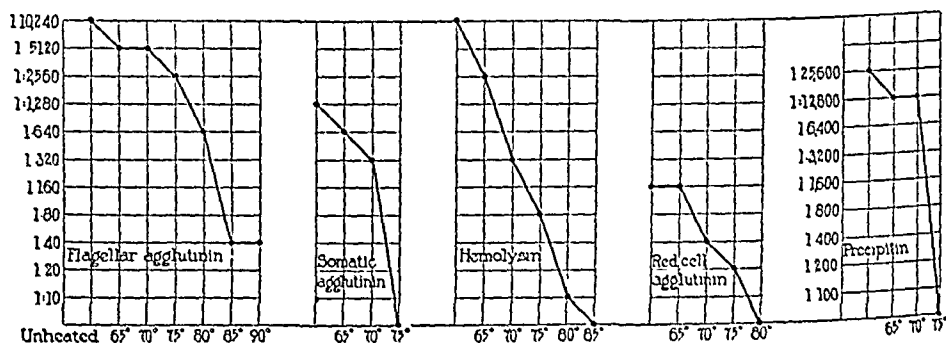
It is apparent from the results given in Table III that sufficient flagellar agglutinin, heated to 70°C. for 20 minutes, remains to divert completely the complement. As the temperature is increased less of the complement is diverted, although the inhibition is strong even after 80°C. for 20 minutes. Less of the complement is fixed when the serum is heated to 85°C. and 90°C., although it is apparent that sufficient antibody still remains to influence the intensity of the hemolysis. It can be said that the findings recorded in Table I are confirmed by the results of complement fixation.

It seemed of interest to test various other antibodies under the

same conditions. With this in view, a cow serum precipitin, and anti-sheep hemolysin and red cell agglutinin were subjected to the same method of procedure. In the instance of precipitin the serum was diluted with an equal part of NaCl solution, the hemolysin and hemagglutinin were diluted 1:4.

The effect of heat on precipitin and the persistence of sufficient antibody to bind complement are recorded in Tables IV and V.

It is obvious that the visibility of the precipitin reaction is destroyed when the precipitin is heated to 75°C. for 20 minutes. Complement fixation tests confirm this observation. It might be argued that the comparison between precipitin and the other antibodies is hardly a fair one since the serum mixtures were more concentrated in the



TEXT-FIG. 1. The effect of heat on the various antibodies. Logarithmic curves based on the data given in the tables.

former cases. Such is not the case, since precipitin diluted 1:4 and heated gave the same results in complement fixation tests as that diluted 1:1 and treated in the same manner.

The evidence of the effect of heat on hemolysis and red cell agglutinin is given in Tables VI and VII.

With the increase in temperature the hemolytic titer declines. 65°C. for 20 minutes has an appreciable effect, and increase to 75°C. materially affects the antibody, and only a trace remains after heating to 80°C. A similar result is obtained with the red cell agglutinin except that no agglutinin can be demonstrated in the serum held at 75°C. for 20 minutes.

## DISCUSSION AND SUMMARY.

It is possible by means of curves to depict graphically the behavior of the various antibodies under various conditions. Logarithmic curves based on the data presented in the tables are submitted in Text-fig. 1.

In general it is evident that antibody destruction goes on gradually as the temperature is increased. Thus 65°C. for 20 minutes diminishes the activity of all the antibodies with the exception of red cell agglutinin, and in this case although the final titer was the same evidently some of the antibody was inactivated, since the reaction was weaker in the higher dilutions. It can, then, be said that 65°C. for 20 minutes appreciably affects the activity of all the antibodies tested. When the temperature is increased to 70°C. more marked differences are apparent. Here both types of the bacterial agglutinin and the precipitin are fairly stable when compared with hemolysin and red cell agglutinin. In both instances there is a sharp decline in the activity of the antibody. 75°C., however, is even a more critical temperature since at this point the somatic bacterial agglutinin and the precipitin are completely inactivated. The hemolysin and hemagglutinin behave alike. The flagellar agglutinin is the most resistant of the group to this temperature. When the temperature is increased to 80°C. the red cell agglutinin is completely inactivated, but sufficient hemolysin still remains to give a slight reaction at the lowest dilution. A further increase to 85°C. completely destroyed the hemolysin but left a definite amount of flagellar agglutinin; in fact, 90°C. for 20 minutes did not completely destroy this substance, since well defined clumps in the lower serum dilutions could be detected on microscopic examination. In this respect, then, the observations of Beyer and Reagh and Orcutt that there is a well defined difference between the two agglutinins for the hog cholera bacillus have been confirmed. However, each substance tested, with perhaps two exceptions, differs in its behavior to heat. It is of interest to point out the similarities in the reaction of somatic agglutinin and precipitin. Both are diminished when heated to 65°C.; 70°C. further affects the agglutinin, but not the precipitin; 75°C. completely inactivates both.

The assumption that the substances are apparently destroyed when they cease to react visibly with their respective antigens seems well founded since they cannot be reactivated with normal serum and no longer react to divert complement when combined in a hemolytic system.

It might be of interest to mention briefly other experiments in which the temperature was kept constant and the time varied. Thus temperatures of 50–55°C. and 60°C. maintained for 8 hours had no effect on antibody. 60°C. for 4 days failed to alter materially the flagellar agglutinin, although the same temperature for 24 hours inactivated the somatic agglutinin and the cow serum precipitin. Hemolysin deteriorates slowly at 60°C., so that after 4 days the serum, which originally reacted at a dilution of 1:10,240, only titered 1:160. The red cell agglutinin was about as resistant as the hemolysin in that a little still remained at the end of the test period. The experiments while incomplete add further proof that the somatic agglutinin and the precipitin are the least resistant to heat, while the flagellar agglutinin is on the whole comparatively stable.

A final experiment was performed to determine, if possible, at what temperature rabbit serum globulin was inactivated. With this in view, globulin was obtained by precipitation with ammonium sulfate, and a series of guinea pigs and chickens received several intraperitoneal injections. In no instance was a globulin precipitin obtained. By immunizing fowls in a similar manner with small quantities of rabbit serum good precipitin was obtained. The diluted rabbit sera heated at various temperatures for 20 minutes were tested for their antigenic activity with rabbit serum precipitin. It was found that diluted serum heated to 90°C. for 20 minutes reacted to about the same antigenic level as that not heated. Even boiling for 20 minutes failed to reduce greatly its antigenic properties. Paradoxically the visibility of the reaction was more intense with the antigen heated at the higher temperatures. The phenomenon was altogether so opposed to the usual conceptions of the inactivation of antigens that the subject will be gone into with more detail in a later communication. Although it is not possible to show definitely in the experiments that the globulin is or is not inactivated at certain temperatures, nevertheless it appears

probable that certain of the antibodies are destroyed at temperatures below that capable of greatly altering globulin.

It must be recognized that comparisons cannot be made between similar antibodies in the serum of different species, since somatic agglutinin in rabbit serum resisted 70°C. for 20 minutes, although the same agglutinin in cow serum was destroyed at 65°C.

#### BIBLIOGRAPHY.

1. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903-04, x, 89.
2. Beyer, H. G., and Reagh, A. L., *J. Med. Research*, 1904, xii, 313.
3. Joos, A., *Centr. Bakt., 1. Abt., Orig.*, 1903, xxxiii, 762.
4. Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 627.

Dr. Robert Polig Library

26308





## AGGLUTINATION BY PRECIPITIN.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, May 23, 1927.)

The similarity of the reactions of agglutination and precipitation has been commented on by many. Nicolle (1) combined a watery extract of the typhoid bacillus with other microorganisms or with finely divided particulate matter, and after the addition of a 1:10 concentration of antityphoid serum obtained agglutination of the bacteria or inert particles. Kraus and von Pirquet (2) observed precipitation when specific bacterial antiserum was added to extracts of the organism. Arkwright's (3) experiments were along similar lines. When he added a clear watery extract of the typhoid bacillus to a typhoid culture rendered inagglutinable by washing, and further added dilute acid or diluted antityphoid serum, agglutination resulted. The same effects were obtained when *B. coli* or other organisms or particulate matter were added to the typhoid bacillus extract and mixed with antityphoid serum. Thus he points out that the added bacteria or particulate matter in the presence of the specific extract act in a similar manner to *B. typhosus*. He considers the reactions of precipitation and agglutination analogous.

In the course of other experiments (4) diluted normal rabbit serum was heated at various temperatures and tested with a specific precipitin. On the whole weak reactions were obtained with unheated serum and with serum heated at temperatures up to 70°C. for 20 minutes. When the antigen was heated at 75°C. or higher, the reaction was intensified and at certain dilutions it resembled more nearly agglutination in intensity and amount of precipitate. The results were so striking that they seemed worthy of further investigation.

## EXPERIMENTAL.

Throughout the experiments three precipitins were used. The first few observations were made with a precipitin prepared by injecting fowls with rabbit serum. Later experiments were made with cow serum precipitin and crystallized egg albumin antiserum obtained from rabbits injected with the respective antigens. The methods used are recorded with the experiments reported in detail in the following pages.

TABLE I.

*Effect of Heating Rabbit Serum (Antigen) on Specific Precipitation.*

Rabbit serum (antigen)	Dilutions of antigen						
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
Unheated	+	+	+	++	+	+	+
Heated for 20 min. at °C.							
75	—	—	+	+	++	++	+
80	—	—	+	++	++	++	+
85	—	—	+	+++	+++	+++	+
90	—	—	++	+++	+++	+++	+
Boiled for 20 min.	++++	++++	+++	+++	+++	++	+
Autoclaved, 14 lbs. pressure for 20 min.	+	+	+-	—	—	—	—

\* The bulk of precipitate has been recorded as follows: + + + +, an extremely heavy deposit; + + +, a heavy deposit; + +, less bulky; +, a well defined precipitate; + —, a trace of granular deposit.

It was mentioned that by heating serum antigens the reactions were intensified on the addition of specific precipitin. Inasmuch as these experiments afforded the basis for the whole problem they will be given in detail.

*Experiment 1.*—Rabbit serum was diluted in the proportion of 1 to 4 with 0.9 per cent salt solution and heated at various temperatures for 20 minutes in tightly stoppered tubes in a deep water bath and tested with specific precipitin obtained by immunizing a fowl. Inasmuch as the antigen heated at 60–65° and 70°C. behaved like the unheated serum the results are not given in the table. In the tests 0.1 cc. of the precipitin was added to each tube of diluted antigen. All tubes were incubated for 2 hours and refrigerated overnight. The amount of precipitation is recorded in Table I.

The result was on the whole so unexpected that the experiment was repeated, with similar results. Another experiment in which cow serum antigen was heated and then tested with its specific precipitin yielded similar results. Even boiling the diluted cow serum antigen for 20 minutes served to increase the bulk of the precipitate in the lower dilutions.

It might be argued that the antigenic protein was denatured as the result of heating and that it would react with any foreign serum. To test this possibility the heated rabbit serum was treated with normal fowl serum and the heated cow serum with normal rabbit serum. No reaction occurred in the rabbit-fowl serum series except with the autoclaved antigen, where there was a little precipitate in the lowest dilution. The cow serum boiled for 20 minutes also gave a slight reaction at the lowest dilution when mixed with normal rabbit serum. The results given in Table I must be considered specific.

It was possible to correlate to a certain extent the intensity of the precipitation with the degree of coagulation evidenced by the turbidity of the serum. When rabbit serum is diluted with salt solution and heated at 70°C. it is not greatly altered in appearance. As the temperature is increased the turbidity becomes more marked so that the mixture heated at 85°C. or higher is nearly opaque.

For the purpose of interpreting the results it may be assumed that portions of the diluted antigenic serum coagulate during heating. The mixture then contains in a liquid state unaltered antigen and suspended particles of coagulated protein. It may be further assumed that the coagulated particles are covered with active antigen and when brought in contact with a specific serum may be likened to a bacterial suspension to which specific agglutinin is added. In the first case an agglutination of the protein particles would result when precipitin was added, and in the other bacterial agglutination would result on the addition of agglutinin. The work of many tends to support this hypothesis.

Coulter (5) showed that red cells agglutinate at pH 4.75, but when sensitized with serum the agglutination point was shifted to that of globulin (pH 5.3). Northrop and De Kruif (6) found that a mixture of bacteria and egg albumin or bacteria and globulin behaved toward acid like solutions of the respective proteins; the isoelectric point of the organism shifted to that of the added protein. Loeb (7)



has shown that collodion particles treated with proteins acquire a film of protein on their surfaces. This film causes the particles to assume the character of protein particles in their cataphoretic behavior.

In order to substantiate further the hypothesis that coagulated particles of serum protein are agglutinated on the addition of specific precipitin a further series of experiments was performed.

*Experiment 2.*—If the coagulated particles of protein in the heated serum act as more or less inert material covered with antigen, then the addition to antigen of inert material, such as bacteria or particulate matter, should increase the intensity of the reaction when a specific precipitin is added to the suspension. *B. abortus* was suspended in 0.9 per cent NaCl solution and the turbidity adjusted to 3.5 by the Gates apparatus. This suspension was used as the fluid in which the cow serum was diluted. 0.1 cc. of cow antiserum was added to each tube. The results are given in Table II. A similar observation in which sufficient collodion particles were added to salt solution to make a faintly turbid suspension which was used to dilute the antigen is included in the table. In both series adequate controls containing cow serum and bacteria or collodion particles were tested with normal rabbit serum. For comparison the results of the usual precipitation tests are included.

It is evident from Experiment 2 that the addition of bacteria or particulate matter increases the intensity of the reaction in a manner similar to that observed in Experiment 1 where the antigen was heated sufficiently to cause turbidity. It is of further interest to note that the addition of bacteria or collodion particles produced reactions at higher dilutions. Microscopic examination of the sediment revealed definite clumping of the bacteria and collodion particles.

It will be noted in the experiments thus far that the tests have been conducted as precipitin tests, the antigen has been diluted but the antibody kept constant. It might be argued that, as Arkwright has contended, during the union of antigen and antibody a web is formed and that the bacteria or collodion particles are enmeshed and fall to the bottom of the tube. To show that this is not the proper interpretation of the phenomenon several experiments were performed which conform more closely to the procedure usually employed in bacterial agglutination.

*Experiment 3.*—0.25 cc. of a suspension of collodion particles was added to 5 cc. of normal cow serum. The mixture was incubated for 3 hours and then refriger-

ated overnight. The next day the liquid was drawn off and mixed with an equal volume of normal salt solution. It was centrifuged at high speed and the sediment resuspended in NaCl solution and again centrifuged. The process of washing was repeated twice more. The particles were then suspended in salt solution and tested with cow serum precipitin. Some of the third wash fluid was retained and tested for the presence of cow serum. The results are given in Table III.

If a solution of crystallized egg albumin is substituted for the cow serum and the particles washed three times, resuspended in NaCl solution, and various dilutions of crystallized egg albumin antiserum added, a similar result is obtained, as is shown in Experiment 4.

TABLE III.

*Agglutination of Collodion Particles Sensitized with Cow Serum by Cow Serum Precipitin.*

	Tested with	Amount of test material, in cc.					
		0.1	0.2	0.01	0.002	0.001	0.0005
Collodion particles sensitized with cow serum and washed	Cow serum precipitin	C*	C	C	+++	+	-
	Normal rabbit serum	-	-	-	-	-	-
		1.0 cc.		0.5 cc.		0.1 cc.	
The last wash fluid tested with 0.1 cc. cow serum precipitin.....		+-		-		-	

\* C indicates complete agglutination; + + +, a strong agglutination; and +, a slight agglutination.

*Experiment 4.*—0.4 cc. of a suspension of collodion particles was added to 5 cc. of a 2.5 per cent solution of crystallized egg albumin. After 1 hour's incubation, 5 cc. of normal salt solution was added and the whole reincubated for 30 minutes. The mixture was then centrifuged for a brief interval and the supernatant fluid withdrawn and centrifuged rapidly. The supernatant fluid was discarded and the sediment resuspended in salt solution containing a small quantity of alkali (0.2 cc. N/20 NaOH to 10 cc. NaCl solution). The centrifuging and washing were repeated twice more. The particles were then suspended in slightly alkaline salt solution and tested with crystallized egg albumin antiserum. The results are given in Table IV.

It is evident that the collodion particles attach to themselves sufficient protein, as Loeb maintained, to react in a specific manner

in the presence of specific precipitin. Hitchcock (8) has been able to show that egg albumin adheres to collodion membranes in amounts sufficient to be detected quantitatively.

If it were possible to show that bacteria on coming in contact with proteins retained a film of the antigenic substance which caused them to agglutinate on the addition of a specific precipitin, then the evidence that precipitin and agglutinin were identical would be complete. With this in view a number of experiments were performed. Inasmuch as several species of bacteria react to certain proteins in

TABLE IV.

*Agglutination of Collodion Particles Sensitized with Crystallized Egg Albumin by Crystallized Egg Albumin Precipitin.*

	Tested with	Amount of test material, in cc.					
		0.05	0.02	0.01	0.005	0.002	0.001
Collodion particles sensitized with crystallized egg albumin and then washed 3 times	Crystallized egg albumin precipitin	C	C	C	C	+++	+
	Normal rabbit serum	—	—	—	—	—	—
		1.0 cc.		0.5 cc.		0.1 cc.	
The last wash fluid tested for the presence of crystallized egg albumin with 0.1 cc. of precipitin.....		—		—		—	

a relatively uniform manner, only a single experiment will be reported in detail.

*Experiment 5.*—The growth from a 24 hour agar slant culture of a non-motile strain of the hog cholera bacillus was suspended in 3.5 cc. of normal cow serum which had been previously heated to 65°C. It was then incubated for 3½ hours and an excess of salt solution added and the whole mixed. The mixture was centrifuged rapidly and the supernatant liquid replaced with salt solution. The centrifugation and washing were repeated twice and the bacteria suspended in NaCl solution and tested with cow antiserum. Some of the final wash fluid was retained and likewise tested for cow serum. As a control the same amount of culture was suspended in salt solution, washed twice, and tested with the cow serum precipitin. The results of a typical experiment are recorded in Table V.



It is apparent from the table that a portion of normal cow serum adheres to the bacteria in sufficient quantity to give a characteristic agglutination when mixed with the precipitin. The experiment was repeated with different organisms and comparable results were always obtained. The best results were obtained when the cow serum heated to 65°C. for 20 minutes was used for sensitization. When unheated cow serum is used, the results are about the same; it however usually clumps the bacilli so that aggregates are dealt with and the results for this reason are open to criticism. If serum is diluted 1:5 and used to sensitize the organisms, the reactions are less intense although agglutination occurs on the addition of the precipitin.

TABLE V.

*Agglutination of Bacteria Sensitized with Cow Serum by Cow Serum Precipitin.*

	Cc. of cow antiserum					
	0.02	0.01	0.005	0.002	0.001	Control
Bacteria sensitized with cow serum and subsequently washed twice.....	C	++++	++	+	+-	-
Unsensitized bacteria washed in NaCl solution.....	++	-	-	-	-	-
			1.0 cc.		0.5 cc.	
Last wash solution tested with 0.1 cc. precipitin for cow serum			-		-	

Thus far it has not been possible to sensitize bacteria with various concentrations of crystallized egg albumin. It is true that specific precipitin added to a mixture of bacteria and crystallized egg albumin will agglutinate the bacteria, nevertheless when bacteria are soaked in crystallized egg albumin and subsequently washed they are not agglutinated by the egg albumin antiserum. This experiment was varied in respect to the concentration of egg albumin, pH concentration of the egg albumin, temperature, and time, but in no instance could agglutination with precipitin be obtained after the bacteria had been washed.

## DISCUSSION.

The experiments reported strengthen the belief that the intensity of the reaction when precipitin is added to heated serum antigen is increased because coagulated serum proteins in suspension are covered with undenatured antigen, which under the conditions are agglutinated. The visibility of the reaction is enhanced because of the greatly increased flocculation.

The experiments are of interest in other respects. First the evidence that precipitin and agglutinin are similar is strengthened. When precipitin and its specific antigen are mixed turbidity occurs, later the flocculi increase to the point of visibility and are precipitated. The same reaction can be obtained by mixing bacteria or inert particles with antigen, then adding the precipitin. Here the particles are made up of clumps of bacteria or the inert particles and presumably antigen and antibody. It might be argued that a web similar to that inferred by Arkwright in agglutination was formed in the antigen-antibody union, and that the bacteria were enmeshed in the course of this flocculation. However, if collodion particles were mixed with cow serum or crystallized egg albumin and then washed until free antigen no longer remained in the wash solution, they behaved like bacteria sensitized to cow serum and subsequently washed. The addition of the specific precipitin in increasing dilutions produced agglutination of the bacteria or inert particles. In the experiments of other workers the evidence is presumptive; however, since the antisera employed contained both bacterial agglutinin and precipitin, the presence of both substances complicated the problem. In the experiments here reported a precipitin free of the bacterial agglutinin is shown to behave like bacterial agglutinin.

In a previous communication(4) it was shown that somatic bacterial agglutinin and cow serum precipitin behave in a similar manner toward heat; both are destroyed at 75°C. and both fail to resist 60°C. for 24 hours, although flagellar agglutinin, hemolysin, and hemagglutinin resist these temperatures.

It is of further interest to comment on the behavior of certain proteins under the experimental conditions. If collodion particles are exposed to crystallized egg albumin or cow serum, there occurs a

firm union between the particle and the antigen. Loeb has described this as the deposition of a protein film. He suggested that protein denaturation probably accounted for the deposition of the film. How much denaturation takes place is a question, since in a preliminary report Wu, TenBroeck, and Li (9) state that denatured egg albumin, whatever the agent of denaturation, is immunologically different from egg albumin. The behavior of bacteria toward the two types of proteins is sharply contrasted. There is a definite fixation of the proteins of cow serum to the bacterial cell sufficient to withstand three washings with salt solution. On the other hand, this is not true with crystallized egg albumin. Union evidently occurs, as shown by Northrop and De Kruif (6), but either the albumin is removed by salt solution or so denatured by the bacterial cell that sufficient original protein no longer remains to react when specific precipitin is added. Confirmatory evidence was obtained by means of acid agglutination in that the bacteria soaked in cow serum and then washed agglutinated at about the same acid concentration as a mixture of cow serum and bacteria. Such was not the case when a mixture of crystallized egg albumin and bacteria, and bacteria soaked in the albumin solution and subsequently washed were tested with various concentrations of H ions.

A further series of experiments not reported suggests that precipitin may under certain conditions act as opsonin. If bacteria, antigen, and precipitin are mixed and incubated for 1 hour, then normal rabbit serum and washed rabbit leucocytes added and permitted to act for an hour or more, in the tubes containing bacteria, antigen, and precipitin about three times as many of the leucocytes are found to have taken up the organisms as is the case in the tubes which contain only bacteria plus normal rabbit serum and precipitin or normal rabbit serum and antigen. The difference in the number of organisms per cell is very large; where the immunological series is complete the cells are packed with bacteria, while in the others relatively few organisms are taken up. The results are not so striking as in experiments in which a strongly reacting agglutinin was employed, nevertheless sufficient agglutination and opsonization take place to prepare the bacterial cells for phagocytosis.

It is of interest to note that the experiments tend to corroborate the conception of Avery and Heidelberger (10) that agglutination is a cell surface phenomenon. They point out that the nature of the substance at the periphery of the bacterial cell may determine the readiness of response and even the specificity of the reaction. This seems to be the case when inert particles are coated with crystallized egg albumin or cow serum or bacteria coated with cow serum; the added protein adheres to the particles or bacterial cells and on the addition of the specific precipitin they behave like bacteria in the presence of their specific agglutinin.

#### SUMMARY.

Serum (antigen) when heated at a temperature sufficient to cause definite clouding reacts more intensely with a specific precipitin than a portion of the unheated serum or samples heated at lower temperatures. The phenomenon is explained on the basis that coagulated protein in suspension is covered with undenatured antigen and the addition of precipitin causes agglutination of the coagulated protein. Similar phenomena are obtained when bacteria or collodion particles are mixed with diluted serum (antigen) and precipitin added; the particles or bacteria agglutinate and increase the visibility of the reaction.

Further, it is shown that collodion particles sensitized with cow serum or crystallized egg albumin and subsequently washed until the washing fluid no longer contains the antigenic substance will agglutinate when small quantities of specific precipitin are added. Bacteria sensitized with cow serum and subsequently washed until cow serum no longer remains in the washing solution agglutinate when cow antiserum at fairly low concentration is added. It was not possible to show that bacteria soaked in crystallized egg albumin and subsequently washed retained on their surfaces sufficient undenatured egg albumin to react to crystallized egg albumin precipitin.

#### BIBLIOGRAPHY.

1. Nicolle, Ch., *Ann. Inst. Pasteur*, 1898, xii, 161.
2. Kraus, R., and von Pirquet, C. F., *Centr. Bakt., 1. Abt., Orig.*, 1902, xxxii, 60.
3. Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

4. Jones, F. S., *J. Exp. Med.*, 1927, xlv, 291.
5. Coulter, C. B., *J. Gen. Physiol.*, 1920-21, iii, 309.
6. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 655.
7. Loeb, J., *J. Gen. Physiol.*, 1922-23, v, 395.
8. Hitchcock, D. I., *J. Gen. Physiol.*, 1925-27, viii, 61.
9. Wu, H., TenBroeck, C., and Li, C.-P., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 472.
10. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367.

# LOCAL PROGRESSION WITH SPONTANEOUS REGRESSION OF TUBERCULOSIS IN THE BONE MARROW OF RAB- BITS, CORRELATED WITH A TRANSITORY ANEMIA AND LEUCOPENIA AFTER INTRAVENOUS INOCULATION.

BY CHARLES A. DOAN, M.D., AND FLORENCE R. SABIN, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 8 TO 10.

(Received for publication, May 13, 1927.)

In following the blood of rabbits after an intravenous injection of 1 or 2 mg. of bovine tubercle bacilli, we have found that there develops a characteristic anemia combined with a fall of those white cells that normally arise in the bone marrow. In previous studies, Sabin, Doan and Cunningham (1) presented evidence indicating that the epithelioid cell and its derivative, the giant cell of the Langhans type, come from the monocyte of the tissues. Cunningham, Sabin, Sugiyama and Kindwall (2) then showed that the extension of a tubercular process in the tissues is reflected in the peripheral blood, which they interpreted to mean that from an advancing tubercular area with its massive increase in normal and abnormal monocytes there is a flooding of these cells into the blood stream. Moreover they demonstrated that the healing process involving the increase of lymphocytes around tubercular tissues is also reflected in the blood so that the ratio of monocytes to lymphocytes in the blood stream may be used as an index of the state of a tubercular process in the tissues.

About 2 weeks after an intravenous injection of bovine tubercle bacilli in doses of 1 mg., the percentage and the actual numbers of monocytes in the peripheral blood of rabbits rise. At the same time the monocytes show qualitative changes which involve some lessening of motility and the production of all the intermediate morphological stages between the normal monocyte and the epithelioid cell. The cytoplasmic changes consist in an increase in the number and a de-

crease in the size of bodies, probably vacuoles, that stain characteristically in neutral red in the living cell with a gradual accentuation of the rosette about the centrosphere. In a series of 53 rabbits, Cunningham, Sabin, *et al.*, found that the percentage of monocytes in rabbits infected with bovine tuberculosis rose from an average of 8 per cent before infection to an average of 14 and a maximum of 52 per cent after infection. Their corresponding total numbers were from 943 monocytes per c.mm. before infection to an average of 1455 and a maximum of 6348 after infection. There were no qualitative changes in the lymphocytes but their numbers were increased in rabbits with high resistance to tuberculosis. The normal ratio of monocytes to lymphocytes in the rabbit is 1 to 2.97, in round numbers 1 to 3; rabbits with high resistance developed a ratio of 1 to 3.56 while the animals with low resistance showed a ratio of 1 to 0.79.

The present series of 80 rabbits were infected with 1 or 2 mg. of bovine tubercle bacilli, injected intravenously. We have used the same strain of organisms as in the previous studies, namely Strain B<sub>1</sub>, which was isolated at Saranac but which we obtained from the Dows Laboratory of Tuberculosis, John Hopkins Hospital. Bovine Strain 214 E, secured from Dr. Paul A. Lewis, of the Department of Animal Pathology of The Rockefeller Institute, has given the same findings when used in comparable dosage in a few control animals. Beside studying the white cells in this group we have as a routine counted the red cells and taken the hemoglobin, making the studies on the average of every other day. For the counts of the red cells we have used pipettes and the Levy-Hausser counting chamber calibrated by the Bureau of Standards. The hemoglobin readings were made in a Duboscq colorimeter with the Newcomer standard about 2 weeks after the corresponding counts and by a different individual without knowledge of the totals of the red cells.

These studies have confirmed previous results (2) in demonstrating that in general the progress of the experimental tuberculosis in the tissues can be followed by the ratio of monocytes to lymphocytes in the blood. In studying animals which pass through the acute into the subacute and chronic phases of tuberculosis, it has been possible to follow further the extent of the pulmonary process, and the lesions in other organs, at various stages, and to correlate the changes with the

monocyte-lymphocyte ratio in the blood. In addition, we have found in this study that the curves of the platelets, the red cells, the hemoglobin and the granulocytic leucocytes can in turn be used as a general index of the progress of tuberculosis in the bone marrow.

In the analysis of this series, the animals may be considered in two sections: the first consists of the 12 rabbits studied for the early reaction at 24, 48, 72, 96 hours, and thereafter at intervals of 48 hours up to 18 days; the remaining 68 animals of the series fall naturally into three groups according to the phase of the anemia and leucopenia at the time of death. The progress and extent of the tuberculosis in the early reaction in organs other than the bone marrow are included in a following article (3). While the right femur was chosen for routine examination, these findings were determined to be representative of all the marrow by selected surveys covering both humeri, both femurs and the ribs.

#### *Early Reaction.*

During the first 6 days following the intravenous injection of tubercle bacilli, supravital studies of the bone marrow revealed no changes in the normal picture, except an increase, first noted at 48 hours, in the number (2 to 3 per oil immersion field) and activity of the clasmocytes (3). Fat cells, megacaryocytes, myelocytes, erythroblasts and normoblasts were present in the usual percentage and distribution (11). Only the occasional mature monocyte, to be accounted for by the number to be found in the circulating blood, was seen. On the 6th day, however, there were found (R 217) for the first time, a few monocytes; and on the 8th day the supravital studies (R 218) showed two very definite and striking changes, undoubtedly correlated: (1) changes in the fat cells, and (2) the appearance of many young monocytes.

In this marrow of the 8th day were found, in the supravital studies, later confirmed in sections, a few clumps of fat cells showing the breaking up of the usually large, homogeneous fat globule into smaller droplets, with the beginning of the shrinking of individual cells. The demobilization of fat is obviously the principal mechanism of making room for new elements in bone marrow, as may be seen in the conversion from a fatty to a red marrow under many pathological condi-



ditions. That the fat is in an extremely labile form was demonstrated by one of us (4) in studies in the experimental hypoplasia of avian bone marrow. It was shown that in the pigeon the bone marrow of the radius may be reduced to a completely hypoplastic or fatty state during a fasting period in which there is a loss of from 100 to 150 gm. in body weight. A biopsy at this stage was followed by the giving of abundant food to the bird, with succeeding operative removals of marrow at 24 hour intervals for comparison. In 24 hours there were marked changes in the fat consisting in a regression of the fat cells toward their embryonal state. It is well known that the developing fat cell has the fat in small droplets, the nucleus being centrally placed, the ultimate resting cell showing a flattened peripheral nucleus with one, large, homogeneous fat globule within the stretched cell membrane. The regressing fat cells of the pigeon's marrow showed first a breaking up of the single, large, fat sphere into many smaller droplets. This was followed by the shrinking of the individual fat cells always toward the blood vessels to which they appeared to be anchored, and into which the fat seemed to be passing for transport from the marrow. In 48 hours all the fat had been removed from the pigeon's radius coincident with the restoration of the marrow to a rapidly regenerating hemopoiesis. It was the beginning of such a process of fat demobilization which was evident in the marrow of rabbits on the 8th day after a tubercular infection.

At the same time, the supravital studies showed marked evidence of the development of young monocytes, many of them monoblasts, with rosettes a single granule deep. No epithelioid cells were found. The normal bone marrow does not contain monocytes younger than, nor in numbers exceeding those of the circulating blood, but in this instance as many as 20 per oil immersion field were counted repeatedly. This observation with the living cells was confirmed in sections. In the fixed sections stained with hematoxylin and eosin, it would not have been possible to discriminate the individual monoblasts from myeloblasts with such assurance without the aid of the preliminary supravital studies. Nevertheless there were signs other than staining reaction in the sections themselves which indicated the interpretation to be correct. In normal bone marrow the number of myeloblasts is exceedingly limited; most of the myeloid elements are in the stage of

the late myelocyte, with a full quota of granules, which have been designated Type C (5, 6). Moreover, in myeloid stimulation or in experimental depletion of the bone marrow, myeloblasts do not appear until the myelocytes, Type C, have been reduced and the marrow thrown back to the level of the earlier Types A and B; then such a marrow, if active regeneration is to occur, shows myeloblasts and early myelocytes in mitosis and in increased numbers. In contrast, this marrow of the rabbit 8 days after infection showed only the usual percentage of late myelocytes; thus the myeloid hemopoietic elements were as yet entirely unaffected and the peripheral blood at this stage indicated no change in those blood cells coming from the marrow. Yet in the sections there were large numbers of deeply basophilic, immature forms, with centrospheres easily seen as clear areas in the dense blue cytoplasm. These were the monoblasts and young monocytes which had been seen in the vital preparations. There was no indication that they arose elsewhere than locally in the bone marrow; at this stage there was no rise in monocytes in the blood stream to indicate their transportation through the blood into the marrow. We think that they arose *in situ* from the primitive undifferentiated mesenchymal stem cell which may give rise to any of the three strains of the white blood cells, the leucocyte, lymphocyte or monocyte (6); normally this stem cell, this undifferentiated mesenchyme or so called reticular cell, gives rise only to leucocytes in bone marrow, but, under the stimulus of a tubercular infection, and after the usual very definite latent period (7), the same stem cell may give rise to monocytes, preliminary to the development of the epithelioid cell typical of the specific pathology of the disease.

Chart 1 records the data gathered from the peripheral blood in counts taken before infection and just before autopsy in the 5 animals examined from the 10th to the 18th days of the disease. On the 10th day (R 227) after infection there was a definite decrease in the fat content of the marrow with more evidence of the nature of the demobilization in the number of cells showing the breaking up of the fat into many fine droplets. In addition to increased numbers of young monocytes, some with two nuclei indicating rapid multiplication, there were numerous mature monocytes and the beginning appearance of scattered typical epithelioid cells with a full quota of mitochondria. There was a definite depletion of the late Myelocytes C; Myelocytes B were obvious, but there were no increases in Myelocytes A or in myeloblasts. The red cell series showed a pre-

dominance of erythroblasts, with the appearance of megaloblasts and early erythroblasts. The clasmotocytes remained increased in number with their phagocytic activity stimulated above the normal for this tissue. The megacaryocytes, while not reduced in apparent number, showed here and there degenerating nuclei and cytoplasm.

Supravital studies on the 12th day (R 228) showed markedly decreased fat, many fields having none or only one greatly shrunken fat cell. Every field contained epithelioid cells, either singly or in small clumps; many cells had two nuclei. The myelocytes were thrown back to the B type, only a few C myelocytes being present. The neutrophilic leucocytes in the peripheral blood had fallen

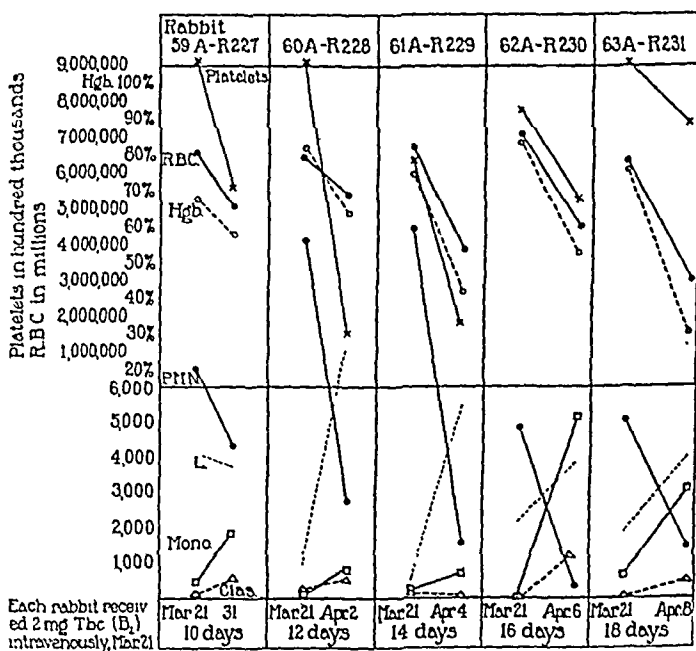


CHART 1.

from an original count of 10,000 per c.mm. to 2800 (Chart 1). The red cells while showing a larger percentage of early erythroblasts than normal still contained normoblasts in appreciable numbers. The clasmotocytes showed engorgement with whole red blood cells. Many of the megacaryocytes appeared shrunken and dead with very little cytoplasm surrounding the nuclei. The platelets had fallen from an original count of 910,000 per c.mm. to 150,000 (Chart 1).

Rabbit 61 A (R 229) (14 days) again showed a bone marrow with the fat absent from many fields in supravital surveys, the presence of fine droplets indicating the continued transportation from the marrow of the final fat deposits. Numerous typical epithelioid cells, many young forms, singly and in clumps, were in every field of every preparation. A marked reduction in C myelocytes, with many

B types, but with the predominating blood cell the early erythroblast, indicated the gradual encroachment of the new growth on the blood-forming tissues. The red cells had fallen from an original level of 6,700,000 per c.mm. to 3,900,000, hemoglobin from 75 per cent to 42 per cent, and the leucocytes from 10,500 to 1580 (Chart 1). There were many clasmotocytes, actively phagocytic, with whole, nucleated and non-nucleated red blood cells and their fragments engulfed. Only two megacaryocytes, one without cytoplasm, were seen in a survey of many fields. The platelets had fallen from an original level of 640,000 per c.mm. to 180,000.

In Rabbit 62 A (R 230) (16 days) supravital studies of the bone marrow revealed practically no fat. There were great numbers of monocytes, typical epithelioid cells and, for the first time, true giant cells of the Langhans type. There was a minimum of myeloid cells at any stage of maturity; the red cell series, largely erythroblastic with a few normoblasts, greatly predominated. The leucocytes had fallen from an original absolute number of 4800 per c.mm. in the peripheral blood to 372, while the monocytes had risen from 0 to 5084 (Chart 1). The clasmotocytes were still stimulated, and the megacaryocytes, though decreased in absolute numbers from the normal, were increasing over the percentage found at the immediately preceding stages.

At 18 days after infection (R 231) the only fat to be found in the bone marrow was an occasional fat globule the size of a red cell. There were many typical epithelioid cells, showing no tendency toward phagocytosis, in sharp morphological and functional distinction to clasmotocytes loaded with phagocytosed white and red blood cells. The monocytes rose from 650 per c.mm. to 3070 in the peripheral blood, and the clasmotocytes from 0 to 465 (Chart 1). There was striking limitation of the myeloid elements, baso-, pseudo- and eosinophilic myelocytes being all proportionately depressed. The red cells showed all stages of erythroblastic maturation, with very few megaloblasts and normoblasts. The red count had fallen from 6,350,000 per c.mm. to 3,030,000 and the hemoglobin from 76 per cent to 30 per cent, the leucocytes from 5000 to 1400. There was a striking excess of megacaryocytes in this marrow in contrast to those marrows of the period from 10 to 14 days. There were many, often nests of 5 per field, small, obviously young cells, with from one to three nuclei; and then, frequently, there would be found large, single, multinucleated cells covering half an oil immersion field. The platelets in the first count before infection in this animal had been 910,000 per c.mm. and in the final count they were 740,000. In the supravital preparation of the peripheral blood the platelets varied greatly in individual size, which variation was quite obvious in the counting chamber also. And this has been confirmed repeatedly in other animals in which the platelets have been followed (see for example Chart 9). During the period of low platelets in the peripheral blood there was a distinctly increased coagulation time for the blood. It will be readily seen from Charts 1 and 9, representative of 14 animals, that the platelets show a sharp decrease in the peripheral blood earlier than the

red or white cells, that the period of depression has a briefer duration (from the 9th to the 14th day after infection approximately) and that the return to the limits of normal comes often while the greatest depression of the other blood elements is being experienced. Coincident with these peripheral manifestations there may be correlated changes in the qualitative and quantitative characteristics of the megacaryocytes in the bone marrow. This evidence might be used in further presumptive corroboration of the direct relationship between the platelet of the blood and the megacaryocyte of the bone marrow, and, conversely, to question further the origin of platelets from the granulocytic and erythrocytic series.

The demonstration of the first appearance of young monocytes in the marrow on the 6th to 8th day after inoculation with massive intravenous doses of bacilli, no epithelioid cells, no tubercles yet having appeared, correlated with the constant development of marked tuberculosis of the marrow following close upon their appearance, is an added confirmation of the origin of the epithelioid cell from and through the monocyte; clasmatoocytes are present in increased numbers from 48 hours on. Since the development of an extensive tuberculosis of the marrow in from 12 to 21 days after infection has been a constant finding with two strains of bovine tubercle bacilli (B<sub>1</sub> and 214 E) (see below), it is believed that the increase in young monocytes marks the onset of the local tubercular process. The fat is gradually depleted as the encroaching tubercular tissue advances and the anemia and leucopenia progress until the beginning spontaneous regression of the foreign tissue makes room within the rigid, bony confines of the hemopoietic organs for adequate blood formation.

#### *The Later Reactions.*

In the remaining 68 rabbits of this series, we have used the preliminary studies of the blood in each animal before infection with tubercle bacilli as the normal base line. The extent of the anemia and fall in neutrophilic leucocytes after infection are indicated in Table I. In this group, the onset of the fall of the red cells and hemoglobin has come on the average 11 days after the injection of tubercle bacilli. This average includes only 62 of the 68 rabbits, because to 6 of the animals had been given a hemolytic serum before the injection of the bacilli, thus introducing two factors in the production of the

anemia. In all 6 there was a further fall of the red cells after the injection of the bacilli but the time of onset was not so striking.

In some instances the fall of the neutrophilic leucocytes (pseudo-eosinophilic in the rabbit) began on the same day as the fall in the

TABLE I.

Blood of normal rabbits. Averages from 68 animals		Average of greatest anemia and fall of P.M.N. in the same 68 animals after infection	
R.B.C.	5,425,697	R.B.C.	2,741,764
Hgb.	62 per cent (Newcomer)	Hgb.	35 per cent
P.M.N.	3833	P.M.N.	940
	44 per cent		17 per cent

TABLE II.

*Age of Cultures of Tubercle Bacilli, Strain B<sub>1</sub>, When Used for Inoculation, with Average Period before Onset of Anemia.*

Age of culture	Average day of onset of anemia
<i>days</i>	<i>day</i>
9	12th
13	9th
14	10th
18	11th
19	14th (All 12 animals)
28	13th
29	11th
32	10th
34	13th
38	11th
56	No anemia produced in 6 animals within 30 days

red cells, but it occasionally preceded the latter, so that the average initiation of the fall in white cells was on the 9th day. The onset of the appearance of this effect on the bone marrow has been quite constant with reference to the subculture used; for example in one instance 12 animals injected from a given subculture all showed the onset of the anemia on the same day; in every group of animals in-

fected from a given subculture 2 or more have developed the anemia simultaneously (Chart 2). There has, however, been no constant relation between the onset of the anemia and the period of incubation of the subculture (Table II); in each instance the culture chosen for inoculation has shown an excellent growth of bacilli and the effective range of incubation has been from 9 to 38 days. With a culture of 9 days the average onset of the anemia was on the 12th day, while with one of 38 days the average onset was 11 days after infection; with one old culture of 56 days there was no manifestation of the development

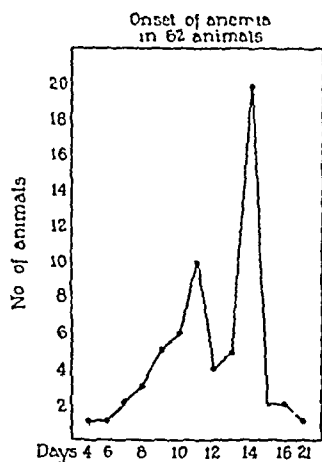


CHART 2.

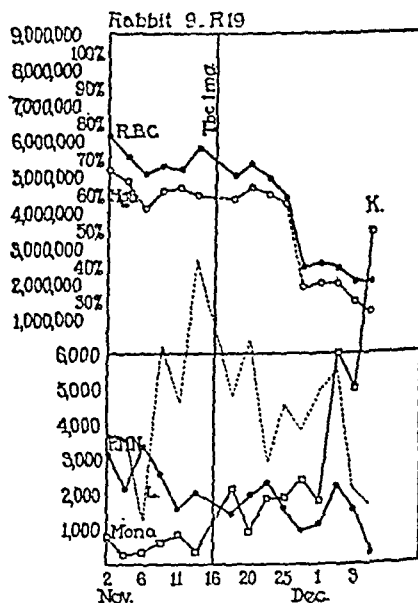


CHART 3.

of an acute phase of the tuberculosis after 30 days in the 6 rabbits inoculated (Chart 5). We have realized that to get the exact time of the onset of the anemia and leucopenia it would be necessary to have counts of the blood every day which was not done in the original series, but the general period of depression has been remarkably constant. In 2 animals of a new series counted every day the onset of the anemia was on the 9th day in both instances and the onset of the leucopenia on the 5th day.

The lowest red count in the series was 1,410,000 cells and the lowest hemoglobin was 21 per cent. There were several instances of an

extreme fall in neutrophilic leucocytes to percentages under 10 and to total numbers ranging from 100 to 200 cells per c.mm. There was one count of 100 white cells with no neutrophilic leucocytes present. The other types of granulocytes, the eosinophilic and basophilic leucocytes, have also shown changes. The eosinophilic leucocytes normally occur in small numbers in the rabbit, ranging from 1 to 2 per cent, and they disappear entirely from the peripheral blood during the leucopenic phase of the disease; the basophilic cells, normally ranging from 8 to 10 per cent, do not decrease so markedly during the leucopenic phase but, as will be shown, they are subsequently increased during the compensatory leucocytosis.

In the analysis of this latter series the animals fall naturally into three groups, according to the phase of the anemia and leucopenia at the time of death. Most of the animals died spontaneously of the disease, but a few were killed when it was estimated that they might not live through the night and supravital studies of the fresh tissues were desired. The first group, 30 animals, showed falling red cells and granulocytes at the time of death, and all died acutely during the 1st month after infection. Of this group 22 died and 8 were killed. The average length of life was 22 days, the extremes being 15 and 34 days. Only 2 of the animals lived longer than 30 days, one 31 days, the other 34 days, and neither of the 2 had a blood count for 4 days preceding death, so that the red cells may have begun to rise in them before death.

The second group consists of 17 animals which showed the beginning of recovery of the bone marrow as reflected by the onset of a rise in red cells, hemoglobin and granulocytes. Of these animals 14 died and 3 were killed. The fatalities came approximately during the 2nd month, with an average length of life of 35 days and a range of from 18 to 60 days after infection; there were 3 animals with a duration of life under 30 days; 1 was killed at 18 days, 1 died at 19 and 1 at 20 days. This group shows the remarkable fact of animals dying with an advancing pulmonary lesion while the bone marrow was recovering from tuberculosis.

The third group consists of 21 animals, in all of which the peripheral blood indicated a restoration of the bone marrow, in that the red cells, the hemoglobin and the granulocytes had either returned to or



exceeded the original normal level. The average length of life in this group was 150+ days, the range of life being from 59 to 247 days, not including 1 rabbit which was infected March 22, 1926, and is still alive after 1 year. Of these animals 14 died, 5 were killed when it was estimated that they might not have lived through the night, 1 was killed while still in good condition and 1 is still living.

On Chart 3 (R 19) is shown the record of the peripheral blood of 1 of the animals representative of the first group. The red cells and hemoglobin are shown in the upper section of the chart, the neutrophilic leucocytes (pseudocoinophilic in the rabbit), the lymphocytes and monocytes in the lower section. Before infection the white cells ranged from 5300 on the 6th of November to 11,500 on the 13th, the increase being due to lymphocytes, not an unusual differential curve in certain normal rabbits; the monocytes were consistently below 1000. As will be seen on the chart, there was a rise in monocytes, which were entirely normal, qualitatively, on the 2nd day after infection. We have found this transitory rise in, usually normal, monocytes on the 2nd or 3rd day after infection in many of the animals of this series. This rise may be due to a division of the monocytes already in the blood stream or to the entry of new cells into the vessels from the tissues, but the survey of the general body tissues during this period (3) reveals primarily a clasmatocyte reaction, so that it would appear more probable as a reaction closely related to the blood itself. From the 1st of December there was a phenomenal rise in monocytes, a large proportion of which were abnormal with lessened motility and with markedly increased bodies staining in neutral red. There were also some typical epithelioid cells. From the 1st to the 4th of December, there was a marked rise in desquamated endothelial cells, clasmatocytes, ranging from 4 to 17 per cent of the white cells in the blood. Two of the endothelial cells from this animal have been illustrated in a preceding paper (9).<sup>1</sup> On December 4th, just before the animal was killed there were several sheets of endothelial cells, one of which contained 19 cells, seen in the peripheral blood, and similar sheets were seen in supravital preparations of the spleen immediately afterward. With the extreme rise in monocytes during the last 3 days, there was a fall in lymphocytes, the final ratio of monocytes to lymphocytes being 75.5 to 14 in percentage and 9475 to 1757 in numbers.

The lines of monocytes and lymphocytes on the chart show the progress of the tuberculosis in the general tissues, while the three lines of red cells, hemoglobin and neutrophilic leucocytes indicate the progress of the lesion in the bone marrow. The abrupt fall in the curve of the red cells came between the counts recorded for the 25th and the 30th, making the drop of the red cells, hemoglobin and the leucocytes come between the 9th and 14th days after infection. In the animals on which a count has been made every day for a comparable period

---

<sup>1</sup> Sabin and Doan (9), Figs. 5 and 11.

the fall has been a gradual one. The curve of the hemoglobin acts as a check on the red cell counts, the close paralleling of the two records indicating the relative accuracy of our present technical methods for ascertaining comparative values at least.

At autopsy the lungs of this animal showed diffuse generalized tuberculosis of so marked a grade that distinct tubercles were not seen in the gross; microscopic sections showed an extreme grade of the disease with only small areas with patent air sacs; there was very little caseation. The spleen was markedly enlarged, weighing 17 gm., the normal average weight being 0.9 gm. according to Rous and Robertson (10); it showed an extreme dilatation of the sinuses and extensive tuberculosis. The free cells obtained by gentle scraping of a freshly cut surface showed some epithelioid cells, large numbers of clasmatoocytes with ingested red cells and many sheets of endothelial cells. On section the spleen showed extreme tuberculosis and markedly dilated sinuses. Our impression is that the marked acute splenic tumor present in every animal of the series that died during the 1st month was due to the dilatation of the sinuses. The lymph glands likewise showed marked tuberculosis with caseation; also dilatation of the lymphatic sinuses. Both glands of the hilum of the lung and the mesenteric lymph nodes were studied. There were a few small tubercles in the liver; the adrenals were negative except for an occasional single epithelioid cell.

The bone marrow of this animal (R 19) is shown in Fig. 1. In the gross the marrow was dark red, elastic, not gelatinous. The striking points in the section are the complete elimination of the fat cells, the extensive tuberculosis and the reduction of the marrow to an early erythroblastic level. The supravital preparations showed no fat, but there were epithelioid cells as evidence of the tuberculosis and early erythroblasts with an occasional megaloblast. In contrast to normal bone marrow, there was a great reduction in myelocytes. A few myelocytes are seen in a gray tone across the artery in the center of the section. The edge of the marrow, which is usually marked by a prominent zone of mature myelocytes, will be seen in this section to be markedly depleted. The section shows two types of areas, the paler zones which are tubercular tissue, the darker zones which are masses of early erythroblasts. There are some megacaryocytes, but our impression is that they are reduced in number. Such a bone marrow corresponds with the peripheral blood on the last day of Chart 3; the leucocytes were reduced to 2.5 per cent with 313 as the total number; the red cells were 2,040,000 and the hemoglobin 27 per cent. In the normal marrow there are many times more myelocytes of Type C (6) than nucleated red cells; in this marrow the supravital studies and the section show vastly more erythroblasts than myelocytes but the erythroblasts were for the most part too young to have been ready for the peripheral blood. Thus this marrow had been depleted of its fat to make room for the tubercular tissue and at the same time the marrow had been depleted of most of its store of myelocytes and normoblasts: it had been thrown back to the level of the early erythroblasts with no sign whatever of a

stimulation of them into the late erythroblasts and normoblasts. Moreover, there was a tendency toward the formation of myeloblasts to indicate a regeneration of the white series. From such a marrow the anemia and the low level of granulocytes in the peripheral blood are readily understood. This marrow stained for tubercle bacilli showed often as many as 10 or 12 acid-fast organisms in the occasional epithelioid cell.

On Chart 4 is shown the blood of a second rabbit (R 18) of the first group. It shows the same points except that the terminal rise in monocytes was not quite so marked though the animal died with the monocytes exceeding the lymphocytes in the peripheral blood. These two charts are entirely representative of the complete group of 30 animals that died in the acute phase of the disease.

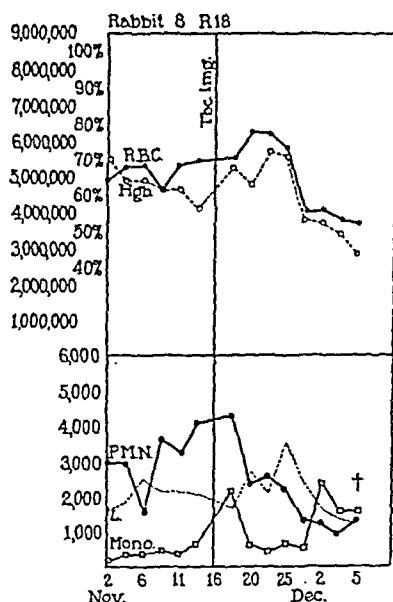


CHART 4.

In every instance there has been extreme or miliary tuberculosis of the lungs; acute splenic tumor with marked tuberculosis; involvement of the lymph glands and extensive tuberculosis of the bone marrow. All the charts of the peripheral blood of this group are practically identical: they show a fall in red cells, hemoglobin and granulocytes which has been correlated at autopsy with the demonstration of a marked tuberculosis of the bone marrow; as a sign of the general tuberculosis, there has usually been the transitory rise in monocytes on the 2nd day, with a marked rise in abnormal monocytes in the 3rd week and a corresponding fall in lymphocytes.

The second group of rabbits, the 17 that survived the first acute hemopoietic depression from the infection, yet died for the most part

in the 2nd month, all showed the beginning of a recovery of the bone marrow.

On Chart 6 (R 80) is the record of the peripheral blood of an animal showing the beginning change from the reaction which occurred in the first group. Before infection this animal received two doses of a hemolytic serum which caused some fall in both red cells and hemoglobin. The blood was counted twice on the day of the injection of the bacilli. At 9.00 a.m. the total white count was 6150; the bacilli were given at 9.30; by 1.30 the white cells numbered 16,500, and the chart shows that the rise was due to a transient outpouring of the neutrophilic leucocytes. The onset of the anemia and the fall in leucocytes secondary to the

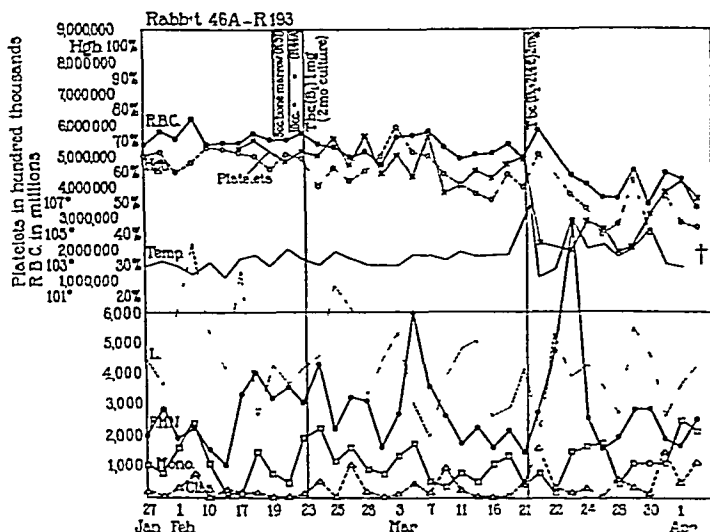


CHART 5.

tuberculosis began on the 9th day. The interesting point in the chart is that there was a slight rise in the red cells, more marked in the hemoglobin just before the death of the animal. A rise in hemoglobin accompanied or followed by a rise in the red cells is constant for the entire group. The terminal rise in leucocytes shown on this chart is unusual for animals dying at this stage. At autopsy, this animal showed extensive tubercles of the lungs, some of which were caseated and surrounded by lymphocytes; this is in contrast to the extreme diffuse reaction of the first group. The spleen had marked dilatation of the sinuses, extensive tuberculosis of the pulp, with some involvement of the follicles. The marrow was most interesting (Figs. 4 and 5). With the low power, the marrow is not far different from that of Fig 1; there is the same extensive tuberculosis, the same

complete elimination of fat and the same reduction of the marrow to an early erythroblastic state. The distribution of the epithelioid cells is rather more diffuse, and this is characteristic of most of the marrows in the early stage; it indicates that there is no reaction whatever on the part of the connective tissue framework in the marrow. The differences between this marrow and that of Fig. 1 are shown in the higher power of Fig. 5 and consist (1) in the signs of degeneration of the single epithelioid cells and (2) in the presence of normoblasts. The large pale epithelioid cells are very clear with the reduced and altered chromatin of the nuclei and the vacuolated cytoplasm. In both marrows there are certain pale areas, from which the epithelioid cells seem to have disappeared entirely as if bone marrow was unfavorable soil for their persistence. There are no signs of

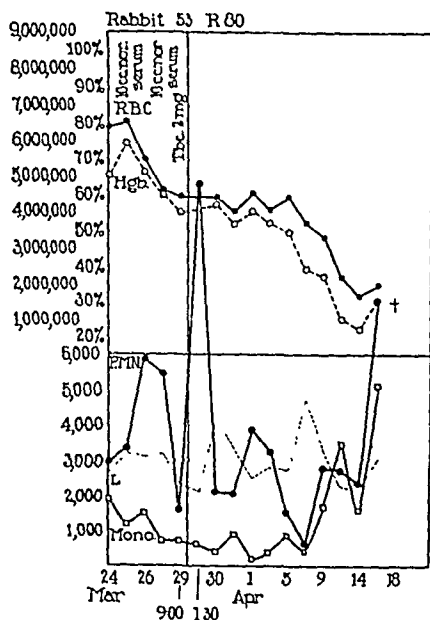


CHART 6.

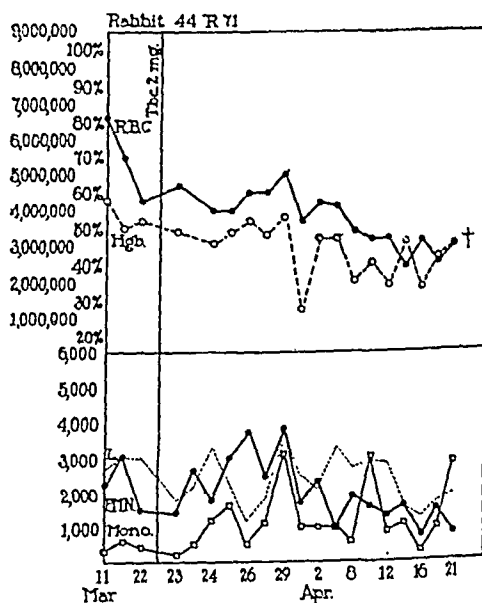


CHART 7.

any degeneration of the white cells, but in Fig. 5 there are two swollen endothelial cells, which have been shown (4, 11) to mark the onset of the regeneration of red cells, and certain lines of normoblasts are quite clear along the left border of the section. It is interesting to note that so slight a change in the red cells as is shown in the chart is readily found in the bone marrow. When this marrow was stained for tubercle bacilli the results were most interesting. Whole bacilli in epithelioid cells were rare. On the other hand the vessels were outlined by cells filled with acid-fast granules in the Ziehl-Neelsen technique. These cells occur along the large venous sinuses, the smaller veins and capillaries and even along the collapsed capillaries which Doan (4) first described as passing between individual fat cells. They make the vascular pattern almost as plain as an in-

jection. The cells with the granules are the clasmatocytes, the so called adventitial cells of Marchand, and occasionally it appears as if the endothelium itself might contain some of the granules. Evidence is presented in a following paper (3) indicating that the clasmatocyte fragments tubercle bacilli in this manner while the epithelioid cell retains them intact.

The same processes, carried a little farther, are shown on Chart 7 (R 71). The corresponding bone marrow is shown in Figs. 6 and 7. The animal lived for 31 days after inoculation and the chart shows a definite rise in hemoglobin, and the very beginning of a rise in the red cells. The lungs had an extreme tubercular pneumonia and no lymphoid reaction. The spleen showed dilatation of the sinuses and tuberculosis of the follicles. In the bone marrow (Fig. 6), it is plain that there are two different types of areas: first, the large tubercular zones such as the one on the left with a few fat cells in the center; second, zones such as the center of Fig. 6 and nearly all of Fig. 7 (higher power) from which the epithelioid cells have disappeared entirely, leaving the normal reticular framework of the marrow. In these latter areas the clumps and lines of developing red cells with their deeply staining nuclei are obvious. Beside the red cells there are small clumps of early myeloid elements, myelocytes, Types A and B, much too young to be giving rise to leucocytes for the blood stream. Again the more advanced regeneration of the marrow is in the red elements as is obvious in Fig. 7, but neither red cells nor white cells are sufficiently advanced to have made a marked change in the blood.

The comparison of Figs. 5 and 7 brings out what happens to tubercular tissue in the bone marrow: the individual epithelioid cells degenerate as shown in Fig. 5 without any evidence of caseation; the debris is quickly cared for as formed and there are left the zones so obvious in Fig. 7 which reveal the reticular framework quite unchanged. In all the marrows, the early erythroblastic group has persisted throughout the acute phase of the disease, only a few megaloblasts having been encountered, that is to say the production of red cells has only rarely been completely thrown back to the level of the parent endothelium, a stage produced in the pigeon by underfeeding (4), and described for the human by Peabody (12). In general, in the animals of our series, the first step in regeneration (excluding the platelets) has been a rise in the hemoglobin, showing that the first compensatory mechanism is an increase in the amount of hemoglobin per cell; the next step is the increase in the number of the red cells and the last the rise in leucocytes. This relative reaction time of red cell *versus* granulocyte follows both the embryonic and the regenerative potentialities of these respective cell types as shown by Sabin (13) and Doan (4, 11).

Further stages in the recovery of the marrow are shown on Charts 8 (R 20), 9 (R 196) and 10 (R 12). On Chart 8 the rise in hemoglobin and red cells is now definite; the leucocytes have stopped falling and are perhaps just beginning to return to the blood. Chart 9 depicts a further recovery in red cells and hemoglobin, with definite recovery in the neutrophilic leucocytes while the animal

was dying of acute pulmonary tuberculosis. This graph is representative of the group of animals in which findings with reference to blood platelets and temperature were studied. In 14 animals in which the platelet count was followed there was uniformly an earlier (average 7 days) and more sudden depression of these elements in the peripheral blood than of the other bone marrow derivatives, though the period of greatest depression usually coincided with a falling red and neutrophil count. The recovery of the platelets both as observed in the peripheral blood and as correlated with the disintegration and regeneration of megacaryocytes in the bone marrow (see Chart 1) indicates a more sensitive mechanism here in its response to the general depression of the disease with a more vigorous and immediate readjustment to the new conditions. In this respect it will be

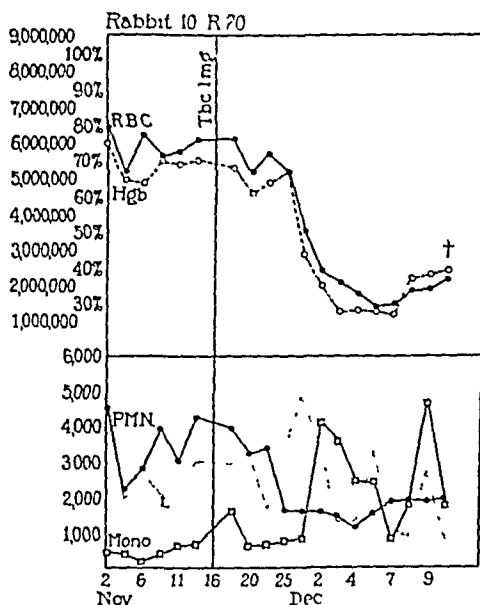


CHART 8.

noted (Chart 9) that the temperature response coincides with the sharp fall in platelets and neutrophils while the red cells are only beginning to show a more gradual decline. The elevation of temperature shows the higher range during the gradual depression of the elements from the bone marrow in the peripheral blood, a period corresponding to the rapid development and extension of the tubercular process in the other organs and tissues of the body as well. Chart 5 (R 193) indicates the lack of elevation of temperature and the uniform blood picture relative to all the bone marrow elements after a 1 mg. injection of bacilli from a culture 56 days old. A reinfection with 2 mg. of a young virulent culture of the same organism promptly initiated the usual changes in the blood with an immediate fall in platelets and a rise in temperature. The clasmotocytic

shower on the day following the reinfection (March 22) is represented by the cell reproduced in another paper (3).<sup>2</sup> This animal died during the acute progression of the tubercular process in the bone marrow. The other 5 animals of this experiment all died acutely within 12 hours after the second reinfection, obviously being in an allergic state even though the blood picture had remained within normal limits and the tubercular process relatively quiescent.

These changes both in the red cells and in the leucocytes are well marked on Chart 10, where the red cells are practically at the original level of between 4000 and 5000. The bone marrow corresponding to Chart 10 is shown in Fig. 2. This animal was in very poor condition, was losing weight and had very sluggish circulation, so it was killed. The lungs showed large and small tubercles sur-

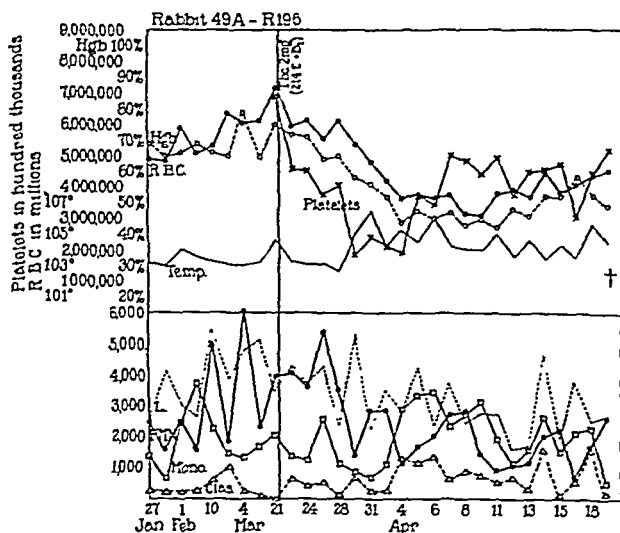


CHART 9.

rounded by lymphocytes. There was very little caseation; no pneumonia. The spleen had very few tubercles; in supravital films most of the free cells were of the clasmatocyte type with but few monocytes. The bone marrow in supravital studies showed the return of the fat; there were large areas of mature myelocytes, some zones of the primitive reticular cells and myeloblasts. Among the red cells were many normoblasts. There is an oblique line across the section to the left of which is a depleted zone from which it is probable that epithelioid cells have just disappeared. There are a few tubercles in the marrow, none showing in this photograph. To the right of the oblique line is an area in which the

<sup>2</sup> Sabin and Doan (3), Fig. 5.



myelocytes predominate over the clumps of nucleated red cells as in normal bone marrow; the area is, however, hyperplastic. To the left of the oblique line the marrow is still considerably depleted and there are patches of younger myelocytes, Types A and B. In both R 20 and R 12 the tuberculosis of the lung was in the form of tubercles rather than the diffuse reaction. The spleen of the animal from which Chart 8 was taken was still enlarged; the one corresponding to Chart 10 was of normal size and contained only a small amount of tubercular tissue.

The last animal of the second group to be illustrated is shown on Chart 11 (R 68), with the corresponding bone marrow in Fig. 3. In this animal it is very clear that the hemoglobin started to rise before the red cells themselves. At the time of death, which was 59 days after infection, the red cells were back to

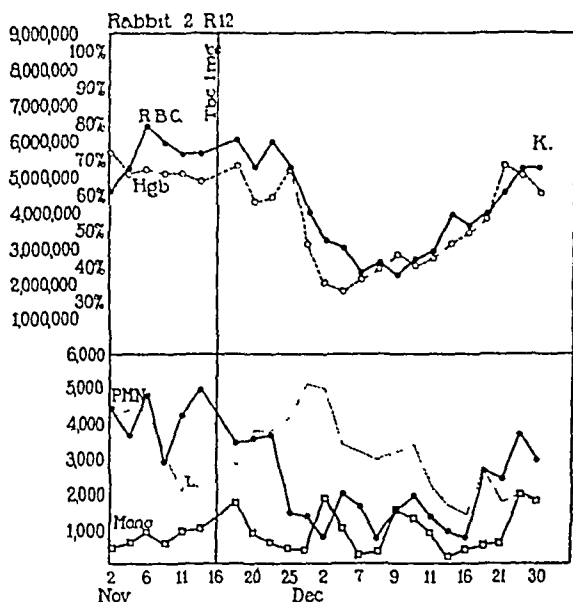


CHART 10.

the original level, while the neutrophilic leucocytes were varying around 6000 in contrast to the original base line of 3000. It will be noted that after the marked rise of monocytes in the 3rd week, they remained high and the lymphocytes dropped, indicating low resistance, and at autopsy both lungs were riddled with tubercles, with large areas of caseation and cavitation. The spleen was of normal size; there were caseous tubercles in the kidneys. On the chart has been added the line of the desquamated endothelial cells, or clasmotocytes. It will be noted that on April 12 the clasmotocytes rose together with the characteristic increase in monocytes of the 3rd week of the disease and then gradually fell to normal numbers. This period of the first great rise in monocytes after massive infection coincides with the period of the extreme diffuse lesions in the lung and

with the acute splenic tumor. At that time there is always the rise both in monocytes and clasmatocytes, together with degenerating types that cannot be analyzed and much cellular debris in the peripheral blood. This is the period of the anemia and the supravital films of the blood always show increased fragmentation of the red cells even when the preparation is looked at immediately and when the temperature of the warm box is carefully regulated so that it does not exceed 37° (14). Beside the free fragments of the red cells in the films of blood, we frequently find some of the clasmatocytes of the blood stream filled with them. Films of the lungs and spleen at this stage always contain clasmatocytes filled with fragments of red cells, the possible stimulus to their increase.

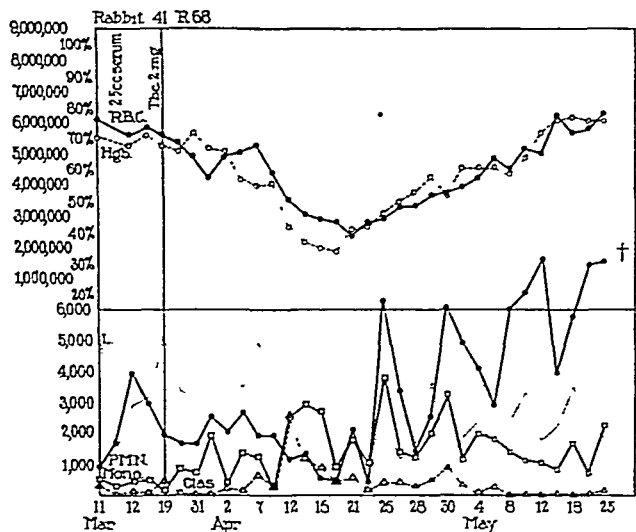


CHART 11.

The bone marrow of this animal (R 68), as will be seen in Fig. 3, is hyperplastic. In the center of the photograph is a small tubercle; the rest of the section shows the gray masses of myelocytes, many of which are still of the younger Types A and B, and the normal proportion of groups of erythroblastic cells. The interesting point in this second group of animals is the universal tendency to a spontaneous healing of the bone marrow of rabbits, irrespective of the eventual fatal progress of the disease in the animal body elsewhere.

When the rabbits survive beyond the 2nd month into the more chronic phase of the disease, the bone marrow passes through the hyperplastic state to the normal. This phase is illustrated in Chart 12 (R 11), and Fig. 8 (R 11), Fig. 9 (R 97) and Fig. 10 (R 95). On Chart 12 is shown a typical picture of the peripheral blood in a rabbit of good resistance; the animal was killed 110 days after

inoculation while still in good condition. In regard to the indications on the chart of the general tubercular infection, there was the usual sharp rise in monocytes on the 2nd day after infection; the monocytes then fell to normal until the 1st of December when there was the characteristic rise of the 3rd week. From the 6th to the 16th of December, the period of greatest leucopenia, it will be noted that the monocytes were again normal and the ratio of monocytes to lymphocytes was entirely normal; this is the period of recovery from the first extreme reaction of the lungs. Then follows the record of a slowly progressing tuberculosis of the lungs in the gradually rising monocytic curve. At autopsy the left lung was largely air-containing; the right was riddled with tubercles, and in sections it appeared that the center of each tubercle was filled with leucocytes

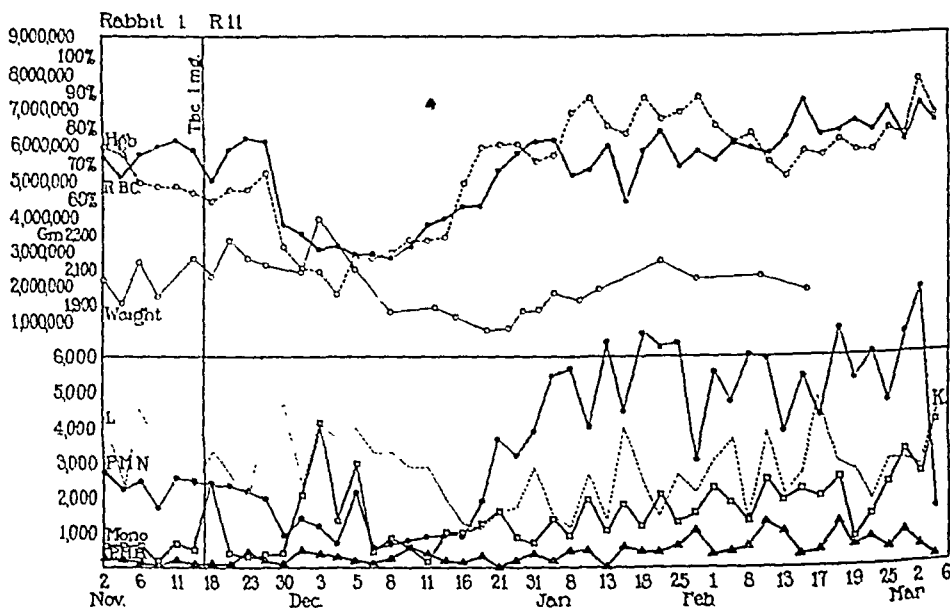


CHART 12.

instead of showing the usual caseation. The liver and spleen did not show any tubercles; there was some myeloid reaction in the spleen. The mesenteric lymph glands were loaded with clasmatoocytes filled with brown pigment but no tubercles were found. In the bone marrow no tubercles were found in sections and no epithelioid cells in the fresh films.

As a record of the functional activity of the bone marrow, it will be seen on the chart that following the period of the anemia and fall of the leucocytes, the marrow recovered its hemopoietic activity and passed into a stage of hyperactivity which was still present when the animal was killed. The lowest line of the chart represents the basophilic leucocytes and shows that though they do not disappear as do the eosinophils during the period of the leucopenia, they, with

the eosinophils, increase during the general hyperplasia of the marrow. In the section of the marrow, Fig. 8, it will be noted that there is a return of the fat cells, though not yet to their normal numbers. No tubercles were found in the sections; the marrow is hyperplastic in still more marked grade than was shown in Fig. 3. There are vast gray areas of myelocytes, largely of Type C with many metamyelocytes. Among the red cells the predominating type was the late erythroblast with normoblasts. The final stage of the disease as far as the bone marrow is concerned involves the complete return to the normal structure with a normal supply of cells to the peripheral blood.

The marrow of Fig. 9 is from a rabbit that lived 135 days and of Fig. 10 from an animal that lived 153 days. In both animals the findings in September after the summer interval showed that the marrow was giving a normal output of red cells and granulocytes to the blood. The rabbit (R 97), from which the marrow of Fig. 9 was taken, had 4,970,000 red cells and a hemoglobin of 61 per cent. The white cells were 11,500, of which the neutrophils were 55 per cent, the lymphocytes 16 per cent and the monocytes 29 per cent. The reversal of ratio of monocytes and lymphocytes is the striking feature. The animal was gaining in weight. The autopsy showed restricted tuberculosis of the lungs but well marked active renal lesions. In all the sections of the bone marrow we found only one tubercle, which is shown in Fig. 9; all the rest of the marrow was entirely normal in appearance. However, in the Ziehl-Neelsen stain the clasmatoctes showed the same acid-fast fragments, though in decreased numbers, as have been described for the early stage.

The rabbit from which the marrow of Fig. 10 was taken also showed a normal output of cells from the bone marrow. The red cells were approximately 6,000,000, the hemoglobin 57 per cent; the neutrophilic leucocytes 47 to 57 per cent; the lymphocytes 9 to 15 per cent and the monocytes 25 to 30 per cent, again a striking reversal of lymphocytes and monocytes. The animal was killed on account of marked tuberculosis of the eyes. The bone marrow appeared entirely normal in the gross and supravital studies, and the sections confirmed this. In this marrow there were still a few clasmatoctes along the vessels containing acid-fast granules.

#### SUMMARY.

In this series of rabbits it was found that the rabbits dying during the 1st month after an injection of 1 or 2 mg. of bovine tubercle bacilli show the same conditions: extreme tuberculosis of the lungs, acute splenic tumor with tuberculosis, involvement of the lymph glands, an occasional small tubercle in the liver and extensive tuberculosis of the bone marrow. The peripheral blood has shown a sharp fall in the platelet count, an anemia and a fall in the granulocytic strain of white cells, and these changes have been correlated with the condition of the

bone marrow. There has also been a rise in monocytes and a fall in lymphocytes, to a reversal of the normal ratio.

When the rabbits have survived the first acute phase of the disease longer than 3 to 4 weeks, there have been signs in the peripheral blood of a recovery of the bone marrow; the first indication of this has been an increase in platelets, then a rise in hemoglobin followed in 1 or 2 days by a rise in red cells and later a return of the three strains of granulocytes. The bone marrow has shown a rapid spontaneous disintegration of the epithelioid cells correlated with the appearance of increased evidence of acid-fast debris in clasmatocytes, especially clear in those that lie along the vessels.

The animals that have survived into the 3rd month have all shown a hyperplastic phase of the healing marrow, both the red cells and all types of the granulocytes appearing in the peripheral blood in numbers above the normal. The epithelioid cells originally containing many bacilli all disappear from the marrow and the only sign left, possibly suggestive of the tuberculosis, is the acid-fast granules in the clasmatocytes. Finally, the marrow becomes entirely normal, giving the normal number of red cells and granulocytes to the blood. Thus, bone marrow in the rabbit has become involved in every instance with the injection of massive doses of viable bacilli. The findings at autopsy in those animals followed during the early reaction to infection confirm this directly and, since the curves of the cells in the peripheral blood of the more chronic animals were the same during the early stages of the disease as in those that died, the same conclusion seems justified from indirect inference for them. The method of healing has been a rapid disintegration of the epithelioid cells without caseation. The bone marrow heals itself entirely regardless of the progress of the disease elsewhere, so that one sees the remarkable condition of an animal recovering from the anemia and leucopenia while dying of tuberculosis elsewhere. The spleen also shows a tendency toward spontaneous healing. In the animals that have lived beyond 100 days there has been some gradual lessening of the diffuse distribution and extent of pulmonary lesions with the development of cavitation together with a marked involvement of the kidneys and lesions in the eyes.

## CONCLUSIONS.

1. With massive intravenous injections (1 to 2 mg.) of bovine tubercle bacilli in rabbits there is a marked involvement of the bone marrow in the early acute phase. This reaction is initiated on the 8th to 10th days by the development of large numbers of young monocytes *in situ*.

2. From the 12th to the 20th day, approximately, there is an increasing development in bone marrow of typical tubercular tissue, epithelioid cells and giant cells of the Langhans type, many showing tubercle bacilli. This new growth eliminates the normal fat cells and encroaches upon and depresses the hemopoietic foci.

3. The bone marrow always tends toward spontaneous healing provided the animals survive the first acute reaction sufficiently long.

4. The method of healing involves a rapid disintegration of the epithelioid cells without caseation and the phagocytosis of debris by the clasmatocytes.

5. The extent and progress of the tuberculosis of the marrow are accurately reflected in the peripheral blood by a decrease of platelets, an anemia and a fall in the granulocytic leucocytes.

6. The onset of the recovery is initiated by the return of the platelets to normal, by a rise in hemoglobin, followed quickly by a rise in red cells, and by a more gradual increase in the granulocytes.

7. During the 3rd month, and after, there is a hyperplasia of the blood-forming elements in the bone marrow with a rise in the peripheral blood of the red cells, hemoglobin and the granulocytes above their original levels.

8. The bone marrow becomes entirely normal when the animal survives beyond 100 days, regardless of a steadily progressing, extensive tuberculosis elsewhere.

9. The varying length of survival in this series of rabbits under uniform environmental conditions, and infected with the same dosage of the same strain of organism, tends to emphasize the importance of the factor of individual resistance of the host in susceptibility to infectious disease.

## BIBLIOGRAPHY.

1. Sabin, F. R., Doan, C. A., and Cunningham, R. S., Discrimination of two types of phagocytic cells in the connective tissues by the supravital tech-

- nique, *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 125.
2. Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., Rôle of the monocyte in tuberculosis, *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.
  3. Sabin, F. R., and Doan, C. A., The relation of monocytes and clasmotocytes to early infection in rabbits with bovine tubercle bacilli, *J. Exp. Med.*, 1927, xlvi, in press.
  4. Doan, C. A., The capillaries of the bone marrow of the adult pigeon, *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 222; The circulation of the bone marrow, *Carnegie Institution of Washington, Pub. No. 277, Contributions to Embryology*, 1922, xiv, 27.
  5. Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A., Studies on the maturation of myeloblasts into myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia, *J. Exp. Med.*, 1924, xl, 845.
  6. Cunningham, R. S., Sabin, F. R., and Doan, C. A., The development of leucocytes, lymphocytes, and monocytes from a specific stem-cell in adult tissues, *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 227.
  7. Sabin, F. R., Doan, C. A., and Cunningham, R. S., The separation of the phagocytic cells of the peritoneal exudate into two distinct types, *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 330.
  8. Cunningham, R. S., Sabin, F. R., and Doan, C. A., The differentiation of two distinct types of phagocytic cells in the spleen of the rabbit, *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 326.
  9. Sabin, F. R., and Doan, C. A., The presence of desquamated endothelial cells, the so called clasmotocytes, in normal mammalian blood, *J. Exp. Med.*, 1926, xliii, 823.
  10. Rous, P., and Robertson, O. H., The normal fate of erythrocytes. I. The findings in healthy animals, *J. Exp. Med.*, 1917, xxv, 651.
  11. Doan, C. A., Cunningham, R. S., and Sabin, F. R., Experimental studies on the origin and maturation of avian and mammalian red blood-cells, *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
  12. Peabody, F. W., A study of hyperplasia of the bone marrow in man, *Am. J. Path.*, 1926, ii, 487.
  13. Sabin, F. R., Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation, *Carnegie Institution of Washington, Pub. No. 272, Contributions to Embryology*, 1920, ix, 213.
  14. Doan, C. A., and Sabin, F. R., Normal and pathological fragmentation of red blood cells; the phagocytosis of these fragments by desquamated

endothelial cells of the blood stream; the correlation of the peroxidase reaction with the phagocytosis in mononuclear cells, *J. Exp. Med.*, 1926, **xliii**, 839.

#### EXPLANATION OF PLATES.

##### PLATE 8.

FIG. 1. R 19, hematoxylin and eosin,  $\times$  about 120. Length of life 18 days. Bone marrow showing extensive, diffuse tuberculosis. Note absence of normal content of fat and myeloid depression.

FIG. 2. R 12, hematoxylin and eosin,  $\times$  about 120. Length of life 44 days. Bone marrow with returning fat cells after regression of the local tubercular process, with hemopoietic hyperplasia.

FIG. 3. R 68, hematoxylin and eosin,  $\times$  about 120. Length of life 59 days. Bone marrow showing one remaining tubercle, without surrounding cellular reaction; marked hyperplasia of myeloid and erythroid foci and beginning re-appearance of fat cells.

##### PLATE 9.

FIG. 4. R 80, hematoxylin and eosin,  $\times$  about 120. Length of life 20 days. Bone marrow with absence of fat cells and depression of hemopoiesis by invading tubercular tissue.

FIG. 5. R 80, hematoxylin and eosin,  $\times$  about 875. Detail of Fig. 4. Beginning regression of the tubercular invasion, showing partition of chromatin in disintegrating nuclei and vacuolated cytoplasm of the epithelioid cells.

FIG. 6. R 71, hematoxylin and eosin,  $\times$  about 120. Length of life 31 days. Bone marrow with local regression of the epithelioid cells showing open areas of regenerating red cells and returning fat even in the midst of intact tubercular areas.

FIG. 7. R 71, hematoxylin and eosin,  $\times$  about 260. Detail of open area in Fig. 6, showing intravascular limitation of developing erythroblasts, a non-cellular matrix marking the former site of invasion of the epithelioid cells.

##### PLATE 10.

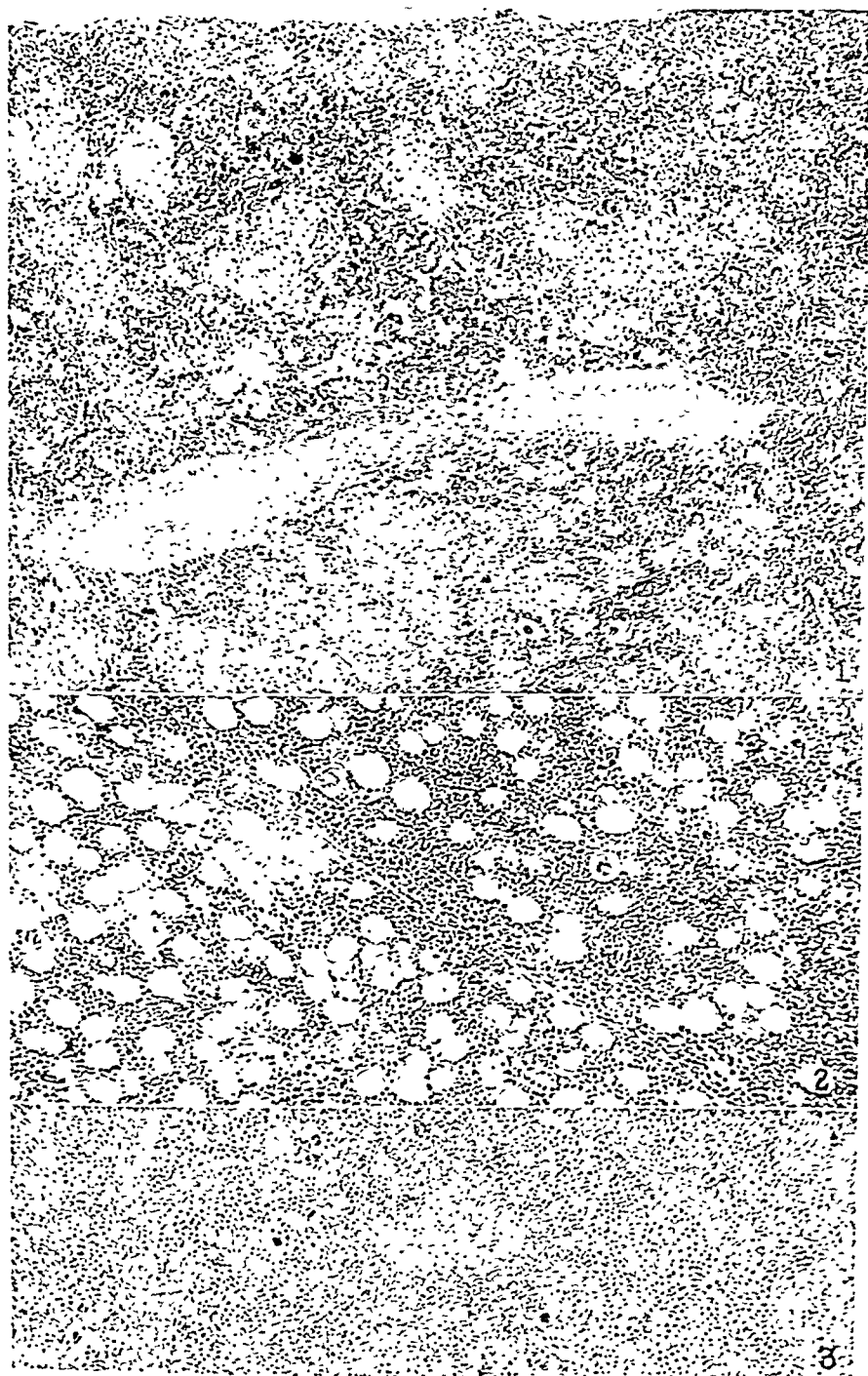
FIG. 8. R 11, hematoxylin and eosin,  $\times$  about 120. Length of life 110 days. Bone marrow hyperplasia of blood-forming elements, no evidence of tuberculosis remaining locally.

FIG. 9. R 97, hematoxylin and eosin,  $\times$  about 120. Length of life 135 days. One tubercle with lymphoid focus in otherwise essentially normal bone marrow.

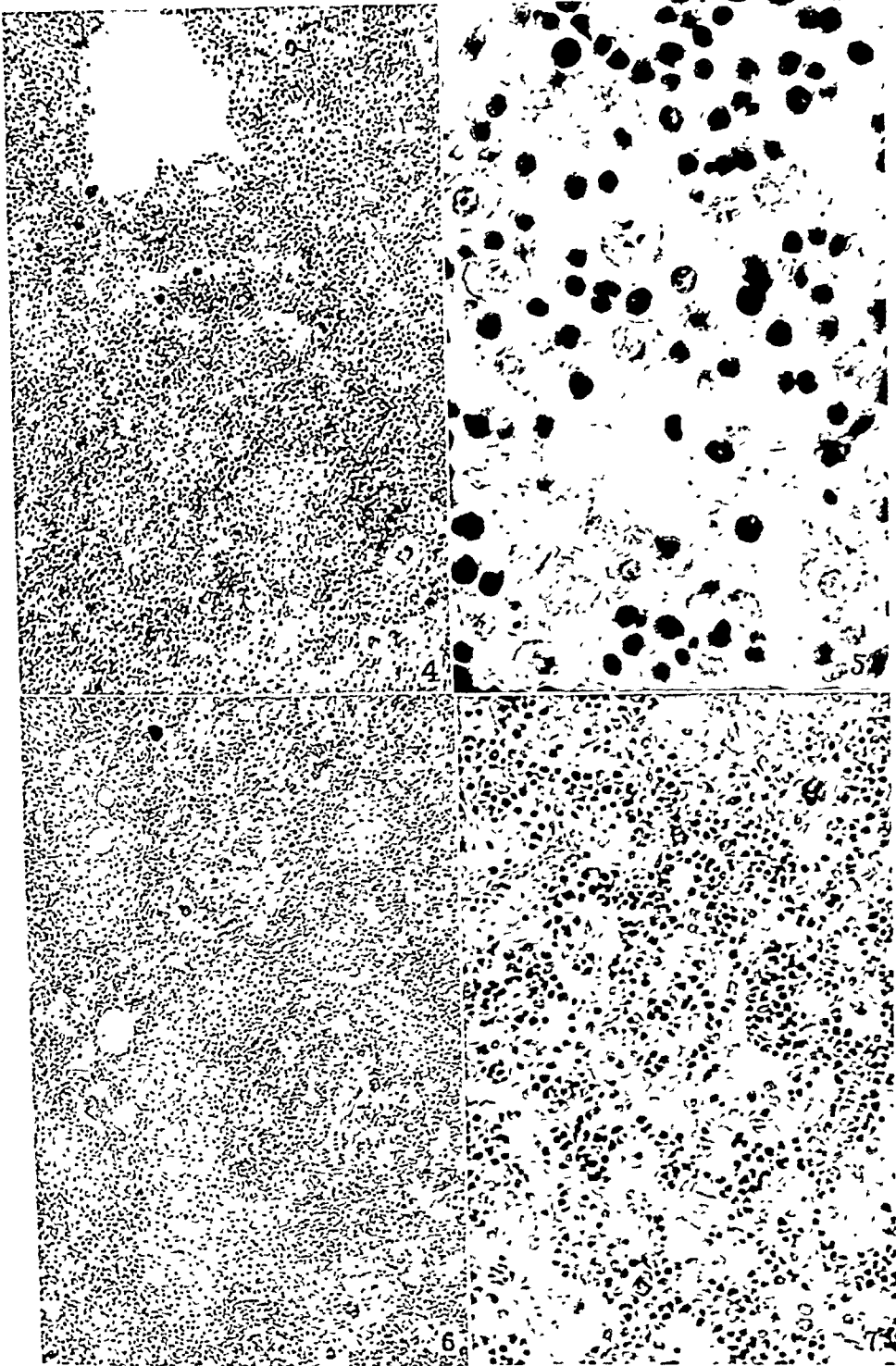
FIG. 10. R 95, hematoxylin and eosin,  $\times$  about 120. Length of life 153 days. Return of bone marrow to normal.



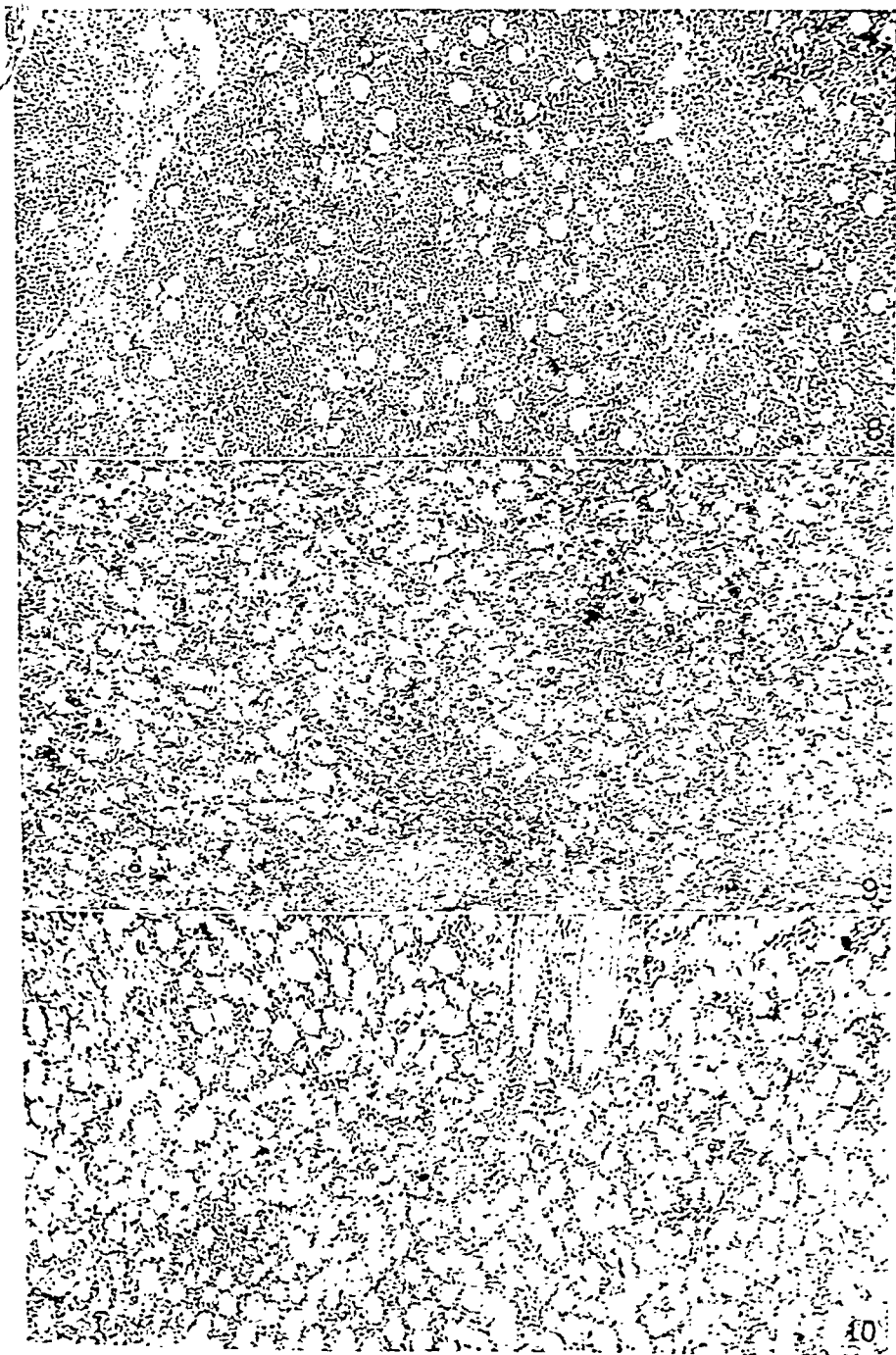














# STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

## III. INCREASED RESISTANCE TO TYPE III INFECTION INDUCED IN RABBITS BY IMMUNIZATION WITH R AND S FORMS OF PNEUMOCOCCUS.

By WILLIAM S. TILLET, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 26, 1927.)

In two preceding papers (1, 2) certain phenomena concerning the antigenicity and infectivity of Type III pneumococci in rabbits were described. The results of these experiments may be summarized as follows: Immunization of rabbits with *Pneumococcus* Type III (1) fails, in the great majority of instances, to stimulate the production of type-specific antibodies, but is always effective in eliciting antibodies reactive with pneumococcus nucleoprotein and R strains derived from all types of pneumococci. These results were interpreted as indicating that normal rabbits possess some mechanism whereby, following the introduction of Type III organisms into the animal body, the antigenic complex of the bacterial cell is so altered that the type-specific component is rendered ineffective as antigen. Since Avery and Heidelberger (3) have shown that type specificity resides in the soluble specific substance which is predominantly present in the capsule, it appears that this altered antigenicity is the result of an injury inflicted upon the capsular substance of the cell. Further evidence in support of this view lies in the fact that *Pneumococcus* Type III possesses low pathogenicity for rabbits. Ten out of eleven strains of this organism, although highly pathogenic for white mice and possessed of large mucoid capsules (S forms), were found to be avirulent for rabbits in doses of 2 to 5 cc. and sometimes 10 cc. (2). Since encapsulation and virulence are generally considered as being intimately associated, it seems possible that the method, whereby



rabbits resist Type III infection, rests on the same mechanism which is responsible for the destruction of the type-specific antigenicity of the cell and that by virtue of the injury inflicted on the capsule of the living organism, virulence is impaired. These results expressed in terms of the host imply that rabbits possess a considerable degree of natural resistance to Type III infection. However, to attribute resistance to natural immunity is merely restating the problem and leaves unexplained the mechanism in the body on which it depends. In the recovery of normal rabbits from Type III infection the evidence indicates that in this instance, the factors underlying resistance are primarily operative against the capsular component of the cell.

The results previously referred to have concerned the reaction of normal rabbits. The experiments reported in this communication deal with immunized rabbits, and the purpose has been to determine the presence of active immunity against Type III infection following immunization not only with homologous organisms but also with heterologous strains, both S and R forms, and with solutions of pneumococci. Others (7, 8) have reported that rabbits immunized with *Pneumococcus* Type III are actively immune against infection with homologous organisms even though specific agglutinins are not demonstrable. These results minimize the rôle which specific antibodies play in active resistance in the case of Type III infection, and Singer and Adler (8) have offered an explanation of this phenomenon to which subsequent reference will be made. It seemed of significance to determine if any demonstrable antibody participated in this active immunity; consequently the sera of all the experimental animals have been tested for both type-specific (anti-S), and species-specific (anti-P), antibodies and the results correlated with the presence or absence of effective resistance.

Testing for the presence of active immunity necessitates the use of virulent organisms. Although most of the Type III strains have been found to possess low initial virulence for normal rabbits, one strain was made highly pathogenic by rapid animal passage. Consequently this rabbit virulent strain afforded a means of testing for increased resistance. Its virulence was maintained so that .001 cc. always proved fatal and, in most of the experiments, .0001 cc. caused death. Since a standard dose of 1 cc. of culture was always injected,

each animal may be considered as receiving usually 10,000 and always 1000 lethal doses.

### *Methods.*

*Antigens.*—Heat-killed cultures, regardless of the type or strain employed in immunization, were, in each instance, made by the same method. 12 to 14 hour plain broth cultures, killed by heating at 56° for  $\frac{1}{2}$  hour, were centrifuged and resuspended in physiological salt solution in such quantities that 0.5 cc. of the vaccine was equivalent to 1 cc. of original culture.

The pneumococcus solutions used for immunization were made by two different methods. One solution consisted of nucleoprotein derived from Type II pneumococcus according to the method described by Avery and Morgan (4). The other solution was made as follows: 6 liters of R<sub>2</sub> culture (derived from Type II pneumococcus) were centrifuged and the bacteria resuspended in 100 cc. of physiological salt solution. 0.3 cc. of 10 per cent sodium desoxycholate was added, and the mixture incubated at 37.5°C. for 2 hours. At the end of this time stained films of the fluid showed a complete dissolution of all formed cells. The solution was then centrifuged at high speed to remove detritus, and the supernatant fluid filtered through a Berkefeld V filter. The bacteria-free filtrate was used for immunization.

*Methods of Immunization.*—All rabbits receiving heat-killed organisms were immunized according to the method described by Cole and Moore (5), which consists in alternating for 6 weeks, a week of daily injections of 0.5 cc. of vaccine followed by a week of rest.

Rabbits injected with pneumococcus solutions received 0.5 cc. intravenously each day for the 1st week and during the 2 other alternate weeks of injection received 1 cc. daily.

All animals were bled 8 to 10 days after the last injection and the antibody content of the sera determined.

*Method of Testing Active Immunity.*—As previously stated, a standard dose of 1 cc. of culture of the rabbit virulent strain of Type III was intravenously injected in each test animal. .0001 cc. of this culture was usually fatal and .001 cc. always killed normal rabbits.

For purposes of following the course of the blood infection in both immunized animals and normal controls, blood cultures were taken at frequent intervals according to the method previously used (2). 4 to 6 rabbits in addition to 1 or 2 controls were tested simultaneously.

Most of the animals which died were examined post mortem with special reference to gross pathological changes in the serous cavities.

Altogether, 44 rabbits have been tested for the presence of active immunity against infection with a rabbit virulent strain of Pneumo-

coccus Type III. In addition to animals which, by surviving, demonstrated a solid immunity, others were considered as partially immune, which, although ultimately succumbing, showed evidence of increased resistance by reason of the duration of life, the character of the bacteraemia as contrasted with controls, and the presence of localized infection found post mortem. A more detailed consideration of these factors will be given in the analysis of the results.

The experimental animals, depending upon the pneumococcus material used for immunization, may be grouped as follows:

- I. Rabbits immunized with Pneumococcus, Type III.
- II. Rabbits immunized with Pneumococcus, Type I or II.
- III. Rabbits immunized with Pneumococcus, R strains.
- IV. Rabbits immunized with solutions of Pneumococcus.

*Group I. Rabbits Immunized with Type III Pneumococcus.*

Twelve rabbits belong to this group. They were chosen from the 28 rabbits used in the immunization experiments previously reported (1). The strain of Type III used for immunization of these animals, although encapsulated, type-specific, and highly pathogenic for mice, was avirulent for rabbits in doses of 5 cc. 3 of the 12 possessed type-specific agglutinins in low titre (2 of the sera were not reactive beyond 1:2 dilution and the other was not reactive beyond 1:20 dilution). The sera of the other 9 contained no demonstrable type-specific antibodies. The sera of all the animals possessed antiprotein antibodies, and agglutinated R strains in 1:160 or 1:320 dilutions of serum.

Of the 3 rabbits whose sera possessed demonstrable type-specific antibodies, 1 survived, 1 lived 7 days and showed evidence of increased resistance, 1 died simultaneously with the control. Of the other 9 rabbits, 5 survived, 3 lived 11, 16, and 22 days respectively showing evidence of increased resistance, 1 died with the control.

In summary, it may be seen that of the 12 rabbits immunized with Type III pneumococcus; 6 survived, 4 showed evidence of increased resistance, and 2 died at the same time as the controls. These results expressed in percentage are: 50 per cent survived and, by including the partially immune, 83 per cent showed definite evidence of increased resistance.

*Group II. Rabbits Immunized with Type I or Type II Pneumococci.*

Fifteen rabbits belong to this group; 10 were immunized with Type II, 5 with Type I, and all were subsequently infected with Type III. The sera of these animals contained homologous type-specific agglutinins to the usual degree and also antiprotein antibodies agglutinating R pneumococci to approximately 1:160 dilution of serum. Of the 10 rabbits immunized with Type II, 7 survived, 2 living 6 and 7 days respectively, were considered as possessing some immunity, 1 was unprotected. Of the 5 rabbits immunized with Type I, 4 survived and 1 was unprotected. The results obtained with rabbits immunized with heterologous type-specific pneumococci may be summarized as follows: 11 survived infection with Type III, and 2 showed evidence of increased resistance. Expressed in percentage, 73 per cent survived and a total of 86 per cent showed evidence of active immunity.

*Group III. Rabbits Immunized with R Forms of Pneumococci.*

Eleven rabbits belong to this group. 5 were immunized with an R strain derived from Type I pneumococcus (designated R<sub>1</sub>); 5 with an R strain derived from Type II (designated R<sub>2</sub>); 1 with an R strain derived from Type III (designated R<sub>3</sub>). The sera of these rabbits contained no type-specific antibodies, but only antiprotein antibodies reactive with R strains in dilutions of 1:160 to 1:320. Of the 5 rabbits immunized with R<sub>1</sub>, 3 survived, 1 lived 6 days with evidence of resistance, and 1, living 4 days, was classified as not being immune. Of the 5 immunized with R<sub>2</sub>, 4 survived, and 1 lived 5 days with evidence of resistance. The 1 animal immunized with R<sub>3</sub> survived. The results with the rabbits immunized with non-type-specific, non-encapsulated R strains show that 8 survived, 2 showed evidence of increased resistance, and 1 was not considered as immune. Expressed in percentage, 72 per cent survived and a total of 90 per cent showed evidence of increased resistance.

*Group IV. Rabbits Immunized with Solutions of Pneumococci.*

Six rabbits belong to this group. The sera of these animals contained only antiprotein antibodies. The titre of R agglutinins obtained by immunization with purified nucleoprotein (Rabbits 24 and

25 in Table III) was 1:100. The antiprotein antibodies present in the sera of the rabbits immunized with desoxycholate solution of pneumococci (Rabbits 27, 28, 29, and 30) agglutinated R organisms in dilutions of 1:1260. No type-specific antibodies were demonstrable.

None of these animals showed evidence of increased resistance to the amount of culture with which they were infected. Although 1 lived  $3\frac{1}{2}$  days, blood cultures showed countless organisms ( $\infty$ ) in each culture taken later than 18 hours after infection. Table III shows the course of the bacteremia in this group of animals, which is in striking contrast to the blood infection occurring in resistant animals (Tables I and II).

*Analysis of Results.*—The data presented in the foregoing experiments reveal the interesting fact that of 38 rabbits which had been previously immunized with pneumococci 25 survived the intravenous injection of living, virulent Type III organisms in amounts at least a thousandfold greater than the dose invariably fatal for normal controls. A second point of equal interest is the fact that this solid immunity against Type III infection may be established by preliminary treatment with cells of heterologous types and with the degraded R forms of pneumococci. In other words, this form of active immunity is effective in the complete absence of demonstrable type-specific antibodies in the serum of the treated rabbits, and appears to be unrelated to the variety of pneumococcus used for immunization. In attempting to analyze the mechanism underlying this form of effective but non-specific resistance recourse was had to the method previously described (2) by means of which the course of the infection may be followed by observing the intensity and duration of the bacteremia. The course of the bacteremia in 7 of the infected rabbits is given in Table I. The figures presented in the table are typical and representative of the results obtained in the group of animals surviving infection, regardless of the variety of pneumococcus used for immunization, and are equally characteristic of the normal control group. 3 of the animals whose protocols are given, had been previously immunized with a rabbit avirulent strain of Type III, 3 others had been similarly treated with R cells derived from heterologous types, and the remaining rabbit had been immunized with Pneumococcus

Type II. For purposes of comparison the course of the bacteremia during the fatal infection of 2 normal control rabbits is included in the same protocol. The rapidly fatal septicemia in the non-immune animals with progressively increasing and overwhelming numbers of organisms constantly in the blood is in striking contrast to the mild,

TABLE I.

*Course of Bacteremia in Immunized Rabbits Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.*

(The results given in this protocol are representative of 25 rabbits which survived.)

Rabbit No.....	1	2	3	4	5	6	7	8	9
Pneumococcus used for immunization	Normal control	Normal control	Type III	Type III	Type III	Type II	R <sub>1</sub> strain	R <sub>1</sub> strain	R <sub>2</sub> strain
Time of culture	No. of colonies per unit of blood								
15 min.	∞	∞	49	660	164	261	3200	620	119
2 hrs.	107	∞	1	2	0	12	22	0	37
5 "	393	∞	0	0	3	5	6	1	20
12 "	562	∞	0	6	0	9	12	8	
24 "	∞	D	0	18	0	38	18	3	216
36 "	∞		0	0	4	72	6	10	12
48 "	D		0	0	23	4	5	176	32
72 "			0	0	14	0	5	14	4
4 days			0	0	30	29	3	342	35
5 "			S	S	7	14	15	66	5
6 "					4	6	1	8	6
7 "					7	2	0	6	0
8 "					2	3	6	4	0
9 "					4	0	0	2	0
10 "					1	0	0	0	0
11 "					0	0	0	0	0
					S	S	S	S	S

S indicates survival of animal.

D indicates death of animal.

fluctuating, but progressively decreasing bacteremia which characterizes the course of infection in the immunized rabbits. In many instances, the blood infection in the immune animals may persist for several days, the circulating organisms varying in number from time to time before ultimately disappearing. The non-fatal course of the bacteremia in the treated animals following infection with an S strain

of Type III, highly virulent for rabbits, parallels very closely the curve of the benign bacteremia (2) which occurs in normal rabbits infected with an S culture of Type III, avirulent for this species. The

TABLE II.

*Course of Bacteremia in Immunized Rabbits Not Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.*

Rabbit No.....	10	11	12	13	14	15	16	17	18	19	20	21	22
Pneumococcus used for immunization	Normal controls		Type III	Type III	Type III	Type III	Type III	Type III	Normal controls		R <sub>2</sub>	R <sub>1</sub>	R <sub>3</sub>
Time of culture													
15 min.	∞	∞	29	168	∞	184	330	164	∞	∞	268	4000	564
2 hrs.	∞	3200	9	253	96	15	3	1	3000	∞	4	28	3
5 "	∞	∞	2	406	13	22	3	0	∞	∞	3	5	5
12 "	∞	∞	5	592	24	90	38	0	∞	∞	0	7	9
24 "	∞	∞	6	D	8	15	314	0	∞	D	34	2	2
36 "	D	D	1		4	D		0	D		103	83	5
48 "			251		12		328	23			316	41	4
72 "			133		3		19	14			56	9	24
4 days			316		8		42	30			38	D	33
5 "			93		12		35	7			D		123
6 "			116		216		21	4					D
7 "			D				1200	7					
8 "					72		86	2					
9 "					1500			4					
10 "					D		10	1					
11 "							4	0					
12 "								2					
13 "								1					
14 "							20	6					
15 "							32						
16 "							D						
17 "								3					
18 "													
19 "													
20 "								1					
21 "								D					

D indicates death of animal.

possible significance of the similarity in the course of the bacteremia in both instances and its relation to the mechanism of recovery will be discussed later.

Of the 38 rabbits immunized with pneumococci—25 of which completely recovered from virulent Type III infection—there were 13 animals which died. However, 9 of these may justly be considered as having acquired a considerable though ineffective degree of immunity as a result of the previous immunization. These animals lived 5 to 21 days following infection, whereas the controls all died within 24 to 36 hours. As previously stated, all the experimental animals suffered a massive infection receiving 1 cc. of the virulent Type III culture representing from 1000 to 10,000 minimal lethal doses. If the test had been made less severe by giving smaller infecting doses the number of surviving animals would, in all probability, have been greater.

Not only the duration of life but also the degree of the bacteremia evidenced the presence of resistance in these non-surviving animals. In Table II is given the course of the blood infection in 9 rabbits as estimated by blood cultures taken at frequent intervals. From the table it may be seen that there is an initial sharp reduction in the number of circulating organisms in the resistant animals as contrasted with the controls, and that, although death eventually ensued, the blood infection during life ran a moderately low grade and irregular course, not unlike that in the surviving rabbits. Even in 2 animals (Nos. 13 and 15), which died within 48 hours, and not tabulated as immune, the extent of the bacteremia is markedly less than in the controls. The results indicate that these partially resistant rabbits, although not possessing a solid immunity, were capable of checking the infection, either by inhibiting multiplication of the bacteria or by actually destroying them. Still further evidence that the rabbits, in which death was delayed, possessed some immunity is brought out by the fact that at autopsy, of the 9 examined, 7 suffered from purulent pericarditis and pleuritis, a condition not found in the normals, which died of an overwhelming septicemia. Localization of infection is generally considered as evidence of partial immunity and this has been especially emphasized by Stillman (6) in experimental production of lobar pneumonia. It seems highly probable that the local inflammatory processes were at least partially responsible for the fatal outcome.

In striking contrast to the effective resistance against Type III infection acquired by rabbits immunized with pneumococcus cells is



the absence of protection in other animals immunized with solutions of heterologous pneumococci. The course of the infection in this group shows that they possessed no resistance, at least against a dose as great as 1 cc. of virulent culture. 5 of the 6 rabbits died within 48 hours and 1 lived  $3\frac{1}{2}$  days. The bacteremia in these animals was only transiently reduced or entirely unaffected (Table III). Furthermore, none possessed evidence of localization of infection on gross post-mortem examination. It is a striking fact that although these rabbits possessed circulating antiprotein antibodies (anti-P) similar to the rabbits immunized with whole organisms, no increased resistance was

TABLE III.

*Course of Bacteremia in Rabbits Immunized with Solutions of Pneumococci and Injected with Rabbit Virulent Strain of Pneumococcus Type III.*

Rabbit No.....	23	24	25	26	27	28	29	30
Material for immunization	Normal control	Pneumococcus nucleoprotein		Normal control	Desoxycholate solution of pneumococcus (Rs)			
15 min.	∞	∞	∞	∞	∞	∞	∞	∞
2 hrs.	∞	∞	∞	∞	175	∞	115	∞
5 "	714	∞	∞	∞	426	∞	217	∞
12 "	∞	∞	∞	∞	∞	∞	382	∞
24 "	∞	D	∞	D	∞	D	∞	∞
36 "	D		D		∞		∞	D
48 "					∞		D	
72 "					∞			
96 "					D			

D indicates death of animal.

apparent. This is strongly suggestive that anti-P antibodies, in themselves, are not significant in this form of active immunity.

#### DISCUSSION.

The experiments reported in the present paper demonstrate that a considerable degree of increased resistance against virulent Type III pneumococci may be stimulated in rabbits by immunization with homologous or heterologous type-specific S pneumococci or with R forms derived from them. The protection was equally effective regardless of the type of pneumococcal cells used for immunization

(Table IV). The exclusion of a type-specific immune reaction by the use of animals previously treated with heterologous S and R strains, makes it necessary to investigate other factors which might afford an explanation of the active immunity. Singer and Adler (8), in dealing with this problem, concluded that the resistance of immunized rabbits to Type III was dependent upon changes (*Umstimmung*) in reticulo-endothelial cells with which was associated the ability to phagocyte the virulent organisms. Interesting as their experiments were, they were inconclusive in excluding the possible influence of sessile specific antibodies. However, when heterologous S and R

TABLE IV.

*Summary of Results in Rabbits Immunized with Homologous or Heterologous Pneumococci and Subsequently Infected with Rabbit Virulent Strain of Type III.*

No. of rabbits	Immunized with	Type III agglutinins	R agglutinins	No. survived	No. resistant not surviving	Total No. resistant	Per cent	No. not resistant	Per cent
12	Type III	3+ 9—	+	6	4	10	83	2	17
15	{ 10 Type II 5 Type I	—	+	11	2	13	86	2	14
11	R	—	+	8	2	10	90	1	10
6	Pneumococcus solutions	—	+	0	0	0	0	6	100

pneumococci afford protection, as the present study indicates, type-specific immunological reactions are entirely eliminated. Wright (9) in a recent publication has reported the results of extensive studies on pneumococcus immunity. Employing Type I pneumococci he was able to demonstrate active immunity in rabbits although demonstrable agglutinins were not present in the sera of the immunized animals. However, under the conditions of his experiments, he did not obtain increased resistance to Type I by previous injection of heterologous organisms and concluded that the reaction was type-specific.

Although the experiments reported in this paper have been carried out under conditions which entirely exclude type-specific immunity,

nevertheless, the favorable results obtained under such conditions in no sense minimize the thoroughly established significance of type-specific antibodies in protection against pneumococcus infection. Their effectiveness in sensitizing virulent homologous pneumococci and thereby making phagocytosis possible, has been repeatedly observed.

In seeking for an explanation of the form of non-specific immunity against Type III infection on the basis of circulating antibodies it may be noted that the sera of all the immune animals possessed antiprotein (anti-P) antibodies. Antibodies of this character are reactive with the common pneumococcus nucleoprotein (precipitin) and with all R strains (agglutinin). They are not, however, reactive with encapsulated type-specific organisms nor do they confer passive protection on mice against virulent pneumococci. Consequently it is highly improbable that they are responsible for the disposition of pathogenic Type III organisms. More direct evidence of the ineffectiveness of these antibodies in resistance is brought out by the fact that immunization with pneumococcus solutions (derived from heterologous organisms) fails to afford protection although the sera of rabbits so treated possess anti-P antibodies in high titre. The one factor which all the resistant animals had in common was immunization with formed pneumococcal cells. The nature of the material used as antigen rather than the demonstrable antibody response, therefore, seems to be the significant feature in stimulating this form of active immunity.

Since type-specific antibodies have been excluded, and since the presence of species antibodies (anti-P) does not furnish an adequate explanation for the resistance to Type III, it is necessary to seek further for an understanding of this form of immunity. In a previous paper (2) it was shown that rabbits possess a considerable degree of natural resistance to *Pneumococcus* Type III, although the strains used for injection were S forms and highly pathogenic for mice. It was also shown that the blood infection resulting from the injection of these *rabbit avirulent* organisms into *normal animals* is characterized by a prolonged course during which the number of circulating bacteria vary from time to time but eventually disappear. In the present paper it is shown that the bacteremia occurring in an *immune rabbit* injected with a *rabbit virulent* strain runs a strikingly similar course.

Consequently it seems possible that the explanation of acquired resistance, in this instance, is due not to antibodies which have been elicited, but to an increased effectiveness of the mechanism of natural resistance. Wright (9) in his recent publication has offered a similar explanation for the active immunity which he obtained and he considered the difference between normal and immune rabbits to be quantitative and not qualitative. However, the emphasis which he places upon specificity makes it necessary to assume that normal rabbits possess type-specific protective substances, a conception which Sia (10) previously suggested and more recently (11) has further emphasized. The experiments reported in this paper reveal the fact that enhanced resistance to Type III pneumococci may be stimulated in rabbits by previous injections of any *R* or *S* pneumococcus cells, and, according to the explanation advanced, is due to an exaltation of the same factors which endow normal rabbits with natural resistance to Type III infection. Further experiments tending to substantiate this view will be subsequently reported. If this hypothesis proves correct, these experiments also tend to show that, whereas intact cells stimulate the processes of natural resistance, the same material in solution is ineffective, although a similar antibody response (anti-P) is elicited in both instances.

#### SUMMARY.

1. Immunization of rabbits with Type III pneumococci is effective in producing active immunity against infection with a virulent strain of the homologous organism.
2. Immunization of rabbits with Type I or II pneumococci, and with *R* forms derived from any of the fixed types, is equally effective in producing active immunity against Type III infection.
3. Immunization of rabbits with nucleoprotein or with desoxycholate solutions of heterologous pneumococci, under the experimental conditions described, appears to be ineffective in producing active immunity against Type III infection.

#### CONCLUSIONS.

Increased resistance against virulent Type III pneumococci may be stimulated in rabbits by repeated injections of heat-killed cultures

of homologous or heterologous pneumococci. This form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of the pneumococcus used for immunization, is considered dependent upon an exaltation of the same factors which afford normal rabbits natural resistance to Type III pneumococcus.

## BIBLIOGRAPHY.

1. Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 713.
2. Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 1093.
3. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367.
4. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.
5. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.
6. Stillman, E. S., and Branch, A., *J. Exp. Med.*, 1924, xl, 733.
7. Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.
8. Singer, E., and Adler, H., *Z. Immunitätsforsch.*, 1924, xli, 71.
9. Wright, H. D., *J. Path. and Bact.*, 1927, xxx, 185.
10. Sia, R. H. P., *J. Exp. Med.*, 1926, xliii, 633.
11. Sia, R. H. P., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 709.

## STUDIES IN ULTRAFILTRATION.

By HANS ZINSSER, M.D., AND FEL-FANG TANG, M.D.

*(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston.)*

(Received for publication, May 6, 1927.)

The limitations of the term "filtrable virus" have been well defined in a recent critical review on these agents by T. M. Rivers (1), who points out the chaos that has reigned in the loose classification of a variety of pathogenic agents on the uncertain basis of filtrability. His review renders it unnecessary for us to reiterate the difficulties that are involved in any work that deals with the nature of agents that—as he asserts—have never been cultivated in the absence of living cells and can be recognized only by their biological or pathogenic effects. Nevertheless, it would be a considerable step toward clearer understanding could we obtain information concerning the actual sizes of some of those active and pathogenic agents, which pass through filters that hold back the smallest bacteria and which are either too small to be seen with the microscope or, for reasons of chemical constitution, have defied methods of staining.

We shall not refer to the complications that render the process of filtration an uncertain method. Stuart Mudd (2) and others have thoroughly discussed these matters in recent publications, and since our work deals entirely with filtration through collodion membranes, considerations which apply to the ordinary bacterial filters of the Berkefeld, Mandler, Chamberland types are omitted.

Our present communication deals with an endeavour to approach the problem of the magnitude of some of the so called "filtrable viruses" by measuring them against the permeability of graded filters made of collodion by a method more or less analogous to that by which Bechhold (3) attempted to establish a scale of sizes for various substances ranging from crystalloids to Prussian blue. Bechhold used formalin-fixed gelatin and acetic acid collodion filters under considerable pressures. As will be seen, our own methods have aimed particularly at avoiding the high pressures employed by him.

The difficulties of the problem are such that it is of course impossible to arrive at any definitions of actual size, but it does seem to us possible by the methods used to formulate a conception of relative size which may have no inconsiderable biological significance, for the relative size of some of these active agents might well lead to a clarification of disputed points.

Between the smallest colloids and the smallest visible formed living particles there is an enormous range, somewhere within which there may be a transitional stage manifest as the "ferments" and enzymes, substances that partake in some of their activities of the properties of living matter. The difficulty of differentiating between invisible living particles and enzyme-like substances has become one of the recent quandaries of scientific speculation in connection with the so called "bacteriophage" of Twort and d'Hérelle, a difficulty for the solution of which crucial experimental methods are still lacking. It has likewise suggested itself to almost every intelligent worker with viruses such as those of herpes, chicken sarcoma, etc., that it might well be that these agents are not living cells at all, but that all the phenomena in which they are involved could be explained by the assumption of a specific "cytophage" acting upon tissue cells as do the lytic principles of d'Hérelle upon bacteria. Could we with reasonable accuracy compare the relative magnitudes of some of these viruses with known organic and inorganic colloidal suspensions, and with enzymes, the approach to understanding them should be considerably facilitated. Moreover, with knowledge increasing concerning the molecular weights of pure proteins, one might even hope for an eventual definition of the smallest size possible for a living cell capable of metabolic function.

There are, as far as we know, only a few investigations in which methods similar to our own were used for analogous purposes. One of these, by Levaditi and Nicolau (4), published in 1923, consisted in attempts to appraise the magnitudes of rabies virus, encephalitis virus (which they still considered separately from herpes virus) and a "neurovaccine" described by them. Their experiments were irregular and led to conclusions which were entirely at variance with those to which we have been led by our own experience. A later piece of work by Olitsky and Boëz (5) which appeared while we were preparing this article for press, concerns itself, among other things, with the filtration of the virus of foot-and-mouth disease. While their methods were to some extent different from our own, their results indicate a

considerable degree of similarity between the virus of this disease and those which we have studied.

The general technique followed depended upon the production of collodion membranes in which permeability was varied according to the principles worked out by a number of investigators, those particularly consulted by us being Brown (6), Bechhold, Nelson and Morgan (7), Hitchcock (8) and Schoep (9). The principles upon which the variation of the permeability of the collodion membranes depends are as follows:

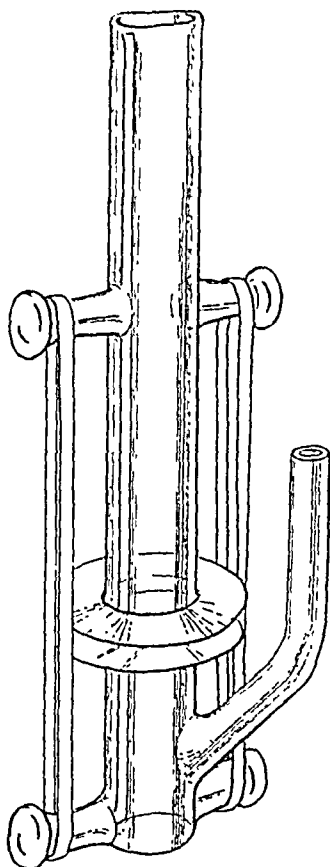
The concentration of the collodion solution; the number of coats of collodion employed; the intervals of air drying between coats; the final drying after the application of the last coat; the percentage of alcohol in which the membrane is soaked after air drying; and the length of time for which it is left in this alcohol solution. This gives a wide range of possible variation, which was at first limited in our experiments by the fact that the use of percentages of collodion less than 1 per cent resulted in membranes too fragile and soft to stand up in the filtering apparatus. This difficulty was overcome as indicated below.

While a number of different methods of carrying out this procedure were from time to time employed, the standard method finally adopted and satisfactorily utilized, throughout, consisted in making the membranes over test-tubes, carefully cleansed, soaked in distilled water and dried. These test-tubes were then slipped over a slightly tilted, motor-driven axis in an apparatus copied in a small form from one used for similar purposes on a larger scale by Dr. Edwin Cohn. The purpose of the motor used was to revolve the test-tube at a uniform rate, while the appropriate collodion solution was being slowly poured over it. Each separate covering of the test-tube in this way was designated a "coat," and a given interval was allowed before the application of a further coat by the same method. After the final coat had been applied and a stated interval again allowed to elapse, the tube was taken off the motor, immersed in diluted alcohol and left there for 30 minutes. At the end of this time it was immersed in water and left there overnight. On the following day the membrane was split with a razor by cutting along a line from one end of the tube to the other. It was then carefully peeled off and floated in water. Each of these membranes could be divided into three or more equal parts for use in the apparatus described below, and usually in our later experiments parts of the same membrane were employed for filtration at one and the same time under the same suction, and tested together for the speed with which they permitted distilled water to pass before and after the actual filtration experiment was carried out.

When it became necessary to produce membranes that would be sufficiently permeable to allow casein to pass through, and collodion



concentrations of less than 1.5 per cent were desired, we found that the resulting membranes were too soft to withstand pressures of 8 cm. of mercury or over. We finally succeeded in producing satisfactory membranes with sufficient permeability and of adequate strength by modifying the above procedure in a simple manner as follows:



TEXT-FIG. 1. The glass apparatus used for the filtrations. It was designed in our laboratory some years ago for another purpose by D. P. Morgan, Jr., and made for us by the Macalaster, Bicknell Company of Cambridge, Mass.

in running water, and with them a permeability could be obtained that was sufficient for our purposes without the extreme friability of the alcohol-ether membranes.

A sheet of Japan lens paper was wound about the cleansed and dried test-tube in a spiral manner, completely covering its surface, and was made to adhere to the test-tube by wetting it down with alcohol-ether, smoothing it and applying it without wrinkles. After this, the collodion coats were applied as usual. This technique, it is found, is useful even for membranes of lesser permeability, since thus reenforced they are more easily peeled off the tubes, and can be more safely manipulated and divided into parts without fracture.

The above technique was quite satisfactory for all filtrations in which permeabilities no greater than those necessary for the passage of collargol and casein were required. When we attempted, however, to obtain a still greater permeability the alcohol and ether method yielded membranes too soft to be manipulated with ease or subjected to any degree of pressure. We carried out our final experiments, therefore, with membranes made by the glacial acetic acid-collodion method described by Bechhold and by Schoep.

Filter paper was impregnated with acetic-collodion *in vacuo*, and the permeability varied by the percentage of gun cotton dissolved in the acetic acid. These membranes were washed for 24 hours

The filtration apparatus used requires no description further than the explanatory legend under the figure included in this paper.

In actually filtering we have attempted to avoid any considerable degree of suction and so to control it that it might be both accurately measured and constant during the experiment. This was accomplished by attaching, to a water pump, a mercury trap which permitted us to vary the suction between 1 and about 20 cm. of mercury, and to hold it for any length of time at any given pressure.

Bechhold, in his work with ultrafiltration, in which he employed filter paper impregnated with gelatin and fixed in formalin or acetic-collodion, used pressures as high as 315 atmospheres and established a graded series of magnitudes for a number of colloidal suspensions. He classifies in this way twenty-three substances, the largest ranging from the crystalloids through various proteins and metallic colloids to Prussian blue. Cohn (10), after determining the minimum molecular weight of certain proteins, has estimated the relative sizes of their molecules by dialysis and ultrafiltration through membranes of graded permeability. By a combination, then, of the analytical and physical-chemical methods, he has constructed a scale in which the molecular weight of egg albumen is 33,400; serum albumen is 45,000 and casein is 192,000. Cohn summarizes the reliability of the filtration method for the determination of the dimensions of proteins by stating that: "If a membrane is made which is permeable to one protein, but not to another, then the latter may be considered the larger, provided the difference is shown not to depend upon the electrical forces involved."

It seemed to us wise to begin our work by comparing a number of the filtrable viruses, namely, that of herpes and the Rous chicken sarcoma, with a typical bacteriophage, with trypsin and with the three proteins—crystallized egg albumen, crystallized serum albumen from horse serum and solutions of purified casein. The crystallized egg albumen and serum albumen (horse) were made by the well known Sørensen method and immunologically determined in the filtrates with specific antisera. Pure casein was obtained from Dr. Edwin Cohn and was determined in the filtrates by means of a relatively weak but specific antiserum and by a ring test in which  $N/40$  hydrochloric acid was layered over the filtrate.

## I.

*Preliminary Experiments.*

Our first membranes were relatively impermeable. They were made by the following formula:

3 per cent collodion, 2 minute interval between coats, 6 coats, 10 minutes final drying, 30 minutes in 40 per cent alcohol, water overnight.

Membranes of this variety in a number of experiments placed in the apparatus under 8 cm. mercury suction allowed egg albumen to pass through but held back horse serum albumen and herpes virus.

A considerable number of experiments of this type were done, and it became clear to us that in developing a satisfactory technique we must take into account changes in permeability that might result from gradual increase of the suction.

An example of such an experiment follows:

*Membrane C12.*—3 per cent collodion,  $\frac{1}{2}$  minute intervals between coats, 6 coats, 10 minutes final drying, 30 minutes in 50 per cent alcohol, water overnight. Filtration at pH 7.2<sup>1</sup>

*Suction cm. Hg—Successive filtrations, same membrane.*

At 3—held back horse serum albumen.

6—let horse serum albumen through.

*(Membrane washed with water.)*

At 3—held back trypsin.

6—held back trypsin.

*(Membrane washed with water.)*

At 6—again let horse serum albumen through.

This experiment indicates that the particular membrane made as described held back horse serum albumen at 3 cm. Hg pressure, but allowed horse serum albumen to pass at a pressure of 6 cm. Trypsin was held back at the higher pressure, and the fact that horse serum albumen passed a second time at 6 cm. after the trypsin experiment had been done indicates that the trypsin was not held back because of the preliminary plugging of the membrane by the serum albumen. This would tend to indicate that trypsin in the impure condition in which we had it was larger than horse serum albumen, always assuming that at pH 7.2 it was not the electrical conditions that determined the permeability.

---

<sup>1</sup> Hereafter, to economize space, we will describe our alcohol-ether membranes by a formula in which the order of the figures follows the sequence of the description of Membrane C 12. Thus, this membrane would be 3 per cent- $\frac{1}{2}$  min.-6-10 min.-30 min. 50 per cent-water overnight.

*Filtration of a Mixture of Serum Albumen and Trypsin Solution.*

*Membrane D 5.*—2.5 per cent— $\frac{1}{2}$  min.—6–10 min.—30 min. 50 per cent.

*Mixture of Trypsin Solution and Horse Serum Albumen.*

pH 7.2 at 4.5 cm. Hg—Horse serum albumen +  
Trypsin —

This experiment again indicates that trypsin in the impure form in which we used it is held back by a membrane which lets through serum albumen.

*Experiment Comparing Horse Serum Albumen and Casein.*

*Membrane E 5.*—1.5 per cent— $\frac{1}{2}$  min.—6–2 min.—25 min. 95 per cent.

*At 2 cm. Hg.*

H<sub>2</sub>O—0.25 cc. in 15 minutes.

Casein solution—0.2 cc. in 15 minutes—negative.

(Washed in water.)

Horse serum albumen—positive.

This experiment indicates that with the membrane made as above indicated casein is held back while horse serum is allowed to pass through. The measurement of the amount of water passed through at a given pressure in 15 minutes, the method adopted throughout in later experiments to determine relative permeability, shows that the casein considerably plugs the membrane; but in spite of this the horse serum albumen still came through after the membrane had been partially plugged by casein. The similarity of the isoelectric points of casein and horse serum makes it unlikely that electrical conditions have any part in the results of the experiments, and indicates that casein is larger than horse serum albumen.

*Experiment Comparing Casein and Trypsin.*

*Membrane E 3.*—1.5 per cent— $\frac{1}{2}$  min.—6–4 min.—30 min. 95 per cent.

*At 5 cm. Hg.*

H<sub>2</sub>O—2.5 cc. in 20 minutes.

Casein—0.35 cc. in 15 minutes—negative.

(Washed with water.)

Trypsin solution—1.25 cc. in 15 minutes +++.

H<sub>2</sub>O—1.0 cc. in 20 minutes.

This experiment shows beyond doubt that trypsin, even in the impure state in which it must have been present in our solution, was smaller than casein. For it is quite apparent that the casein plugged the membrane but that, in spite of this plugging, the trypsin that was subsequently filtered came through readily and the final water test showed that there was no gross leakage.

The foregoing experiments, then, indicate that it is possible to grade filters so that they measure particles in the order in which their molecular weights would indicate size; namely, crystallized egg albumen,

crystallized serum albumen next and pure casein the largest. While these experiments, moreover, indicate definitely that trypsin is smaller than casein, they also suggest that trypsin may be somewhat larger than serum albumen. This, however, cannot be definitely accepted because the trypsin may, in our necessarily impure solutions, be adsorbed to other constituents, possibly to a protein. Nevertheless, it is important in connection with our subsequent studies to realize that in size trypsin probably approximates to serum albumen and is definitely smaller than casein.

## II.

*Filtration of a Staphylococcus Bacteriophage in Comparison with Trypsin and Casein.*

A staphylococcus bacteriophage, which was supplied as through the kindness of Miss Elsie Schumm, was used. To a 5 to 7 hour young culture of a susceptible staphylococcus in broth, 1/10th of the volume of a Berkefeld filtrate of the bacteriophage was added, and the mixture kept at room temperature overnight. On the next morning the culture was found to be clear and the clear fluid was again filtered through a Berkefeld candle and adjusted to pH 7.2 for the experiments. Tests for the phage were then carried out with similar young cultures on the fluid above and below the filter membrane.

*Membrane K 4.*—1 per cent.— $\frac{1}{4}$  min.—6–4 min.—30 min. 50 per cent. Divided into 3 parts simultaneously tested.

	Initial test. Water at 2 cm. Hg in 10 min.	2 cm. Hg 16 min.	Final water test for leakage. 2 cm. Hg 10 min.
	cc.		cc.
K 4a	1.25	Casein 0.8 cc. negative	0.15
K 4b	1.25	Trypsin 0.8 cc. positive	0.25
K 4c	1.25	<i>Staphylococcus aureus</i> bacteriophage 1.5 cc. negative	0.5

This experiment would tend to show that under the conditions of filtration bacteriophage is held up by a filter which permits trypsin to pass.

In this experiment we began a practice which is followed throughout hereafter, and which is of considerable importance. It consisted in not only measuring the flow of water per stated interval at a given pressure for each individual membrane before the filtration of a specific substance was attempted, but in adding to this a final measurement of the passage of water under the same pressure for the same

length of time, after the specific filtration had been carried out. This not only insured us against errors of gross leakages, but furnished a very definite indication of the degree to which the membrane had been plugged by the substance previously filtered. It may be noted in passing that nothing plugged the membranes quite as extensively and regularly as casein.

*Membrane N 5.*—1 per cent— $\frac{1}{4}$  min.—6–4 min.—30 min. 95 per cent—membrane divided into 3 sections.

	H <sub>2</sub> O, 2 cm. Hg 5 min.	2 cm. Hg 6 min.		H <sub>2</sub> O again 2 cm. Hg 5 min.
	cc.			cc.
N 5a	1	Casein	0.75 cc. Casein ++ by acid test	0.25
N 5b	1	Trypsin	1.0 cc. Trypsin ++	0.75
N 5c	1.5 (Bigger surface.)	Phage	1.5 cc. Phage negative	1.0

This experiment shows that under conditions which will let through both casein and trypsin bacteriophage does not pass, even though the tests with water indicated that if there were any difference between the three sections of Membrane N 5, it was in favour of greater permeability for the one through which it is attempted to pass the bacteriophage. Similar experiments confirmed this result with regularity.

### *Filtration of Rous Sarcoma Virus.*

The Rous sarcoma virus was prepared in the following manner: Tumors freshly taken from chicks were minced and ground up thoroughly in sand, then emulsified in Ringer's solution and, in a preliminary filtration, passed through paper pulp and sand filters in the manner usually employed in working with this tumor. The resulting filtrate was the material used in our collodion membrane filtrations.

#### *Comparison of Horse Serum Albumen and Rous Sarcoma Virus.*

*Membrane C 13.*—3 per cent— $\frac{1}{2}$  min.—2–5 min.—30 min. 50 per cent.

#### *Mixture of Rous Sarcoma Virus and Horse Serum Albumen.*

##### *Suction cm. Hg—Filtrations at pH 7.2.*

At 4—held back horse serum albumen.

At 8—held back horse serum albumen.

At 12—held back horse serum albumen.

At 16—let horse serum albumen through.

Chicks were injected with portions of every filtrate except the one at 4 cm. Hg, and all were negative. Controls were positive.

This membrane did not permit horse serum albumen to pass until the pressure was increased to 16 cm. Hg. Even at this pressure, however, chicken sarcoma virus did not pass through the membrane.

*Comparison of Casein and Rous Chicken Sarcoma Virus.*

*Membrane K 5.*—1 per cent— $\frac{1}{4}$  min.—6–2 min.—30 min. 50 per cent—divided into 2 parts simultaneously tested.

	Water at 2 cm. Hg 10 min.	2 cm. Hg 15 min.	Water at 2 cm. Hg 10 min.
	cc.		cc.
K 5a	2	Casein 1 cc. positive test	0.3
K 5b	2	Rous chicken sarcoma 1 cc. chick inoculated negative, control positive	0.5

This experiment indicates that the casein will come through a membrane which holds back the Rous chicken sarcoma. Curiously enough, however, in this case the amount of obstruction of the membrane was greater for the casein than for the sarcoma solution. It is observations of this kind that have suggested to us the necessity of controlling the electrical conditions. Namely, if the negatively charged casein is passed by Membrane K 5, this same membrane might hold up a smaller sarcoma virus if this had an isoelectric point at a pH higher than that at which we were filtering, namely, pH 7.2, and were, therefore, positively charged. This matter is controlled in other experiments below.

*Membrane N 6.*—1 per cent— $\frac{1}{4}$  min.—6–4 min.—30 min. 90 per cent. Divided into 3 parts simultaneously tested.

	H <sub>2</sub> O — 2 cm. Hg	Material filtered	Amount passed in 15 min. at 2 cm. Hg	Retested with water 2 cm. Hg. Amount passed in 10 min.
	cc.			cc.
N 6a	1	Casein solution mixed with Rous sarcoma virus	1.0 cc. Negative for casein Chick inoculated	0.5
N 6b	1	Casein solution alone	1.0 cc. Positive for casein	0.5
N 6c	1.5 (Slightly larger area.)	Rous sarcoma virus alone	1.75 cc. Chick inoculated	1.0

Before filtration these materials all tested by specific anticasein serum and those containing casein found positive. Similar controls were made with the acid test. Chick inoculations both negative. Controls positive in 10 days.

This experiment differs from the others in that we attempted to filter through one part of the membrane a mixture of casein solution and Rous sarcoma virus, filtering them separately through the two remaining sections of the same membrane. The casein tests were done both by anticasein precipitating serum and by layering  $N/40$  hydrochloric acid over the solutions. The anti-precipitating serum showed the casein definitely in the mixture and in the casein solution, but gave no ring in the Rous sarcoma virus alone before filtration, and the acid test showed a sufficient difference between the mixture and the Rous sarcoma virus to be of comparative value.

It is interesting to note that where the casein solution was filtered alone it came through at a pressure that held it back when it was mixed with the Rous sarcoma virus, a phenomenon which in one way or another has appeared with various substances which we have tried to filter in mixtures and for which a number of explanations can be suggested but none definitely proven at the present time.

Again the experiment indicates that the casein particles are smaller than the chicken sarcoma virus. A number of similar experiments gave results of identical significance.

### *Filtration of Herpes Virus.*

The herpes virus used in these filtrations was prepared by taking either a freshly glycerolated or a fresh unglycerolated brain of a rabbit dead of herpes, grinding it thoroughly in sand, taking it up in varying amounts of Ringer's solution and centrifuging for 1 or 2 hours until the supernatant fluid was moderately opalescent. The virus filtrations were done with dilutions of this material. There seems no particular reason for stating dilutions, since on centrifugation such varying quantities of brain material were thrown down that the amounts of possible virus in the supernatant fluid cannot be estimated in any manner that could have any significance.

### *Comparison of Casein and Herpes Virus.*

*Membrane M 2.*—1 per cent— $\frac{1}{4}$  min.—6–4 min.—30 min. 95 per cent. Divided into 2 parts simultaneously tested.

	2 cm. Hg 10 min.	2 cm. Hg 10 min.	4 cm. Hg 5 min.
M 2a	H <sub>2</sub> O 1.25 cc.	Casein solution 0.5 cc. negative	Casein solution 0.3 cc. positive
M 2b	H <sub>2</sub> O 1.25 cc.	Herpes 0.4 cc. Rabbit injected	Herpes 0.3 cc. Rabbit injected

Both animals negative. Control died in 5 days.

This experiment, carried out with two sections of the same membrane simultaneously tested and showing equivalent volumes of water at 2 cm. Hg in 10 min-



utes, indicates that the herpes virus was held back by a membrane which permitted the casein to pass through.

*Comparison of Casein, Herpes Virus and Rous Sarcoma Virus.*

In this experiment three parts of the same membrane were simultaneously tested. In making this membrane, however, the method of dipping in collodion solution was substituted for the rotating test-tube, a fact which probably accounts for the relatively greater permeability of one of these membrane sections over the others. The table shows that K 2a and K 2b let through 1.25 cc. of water at 2 cm. Hg in 5 minutes, while K 2c let through 1.5 in the same time at the same suction. It is apparent from the results that casein came through K 2a at 2 cm. Hg, and that K 2b and K 2c held back both herpes virus and the Rous sarcoma virus at the same suction. The experiment would indicate that the casein particles were smaller than either of the two viruses used, and this result was obtained in spite of the fact that K 2c may have been slightly more permeable than the other two sections.

*Membrane K 2.*—1 per cent— $\frac{1}{4}$  min.—6–2 min.—30 min. 95 per cent. (Dipping method, which accounts for unevenness of membranes.)

	Water at 2 cm. Hg 5 min.	Filtration, 2 cm. Hg 10 min.	
	cc.		
K 2a	1.25	Casein solution 0.8 cc.	Casein positive
K 2b	1.25	Herpes virus 0.8 cc.	
K 2c	1.50	Rous sarcoma virus 1.25 cc.	

Animals inoculated from filtrate of K 2b and K 2c negative. Controls with unfiltered virus suspensions positive.

In the next experiment a freshly made collargol solution,  $\frac{1}{2}$  per cent in distilled water, centrifuged at 2,000 revolutions for 2 hours, was used for comparison. The diameter of the smallest collargol particles so procured is supposed to measure approximately 20  $\mu$ . We have no means of actually measuring these particles, and are accepting the figures of other workers.

*Membrane R 2.*—1 per cent— $\frac{1}{4}$  min.—6–5 min.—30 min. 90 per cent. Divided into four parts.

	H <sub>2</sub> O, 5 min. 2 cm. Hg	15 min. 2 cm. Hg	H <sub>2</sub> O, 5 min. 2 cm. Hg
	cc.		cc.
R 2a	1.0	Collargol 2.25 cc. +++	0.4
R 2b	0.75	Casein 1.0 cc., acid negative, precipitate +	0.15
R 2c	0.75	Herpes 1.25 cc.	0.25
R 2d	1.0	Air test. Air bubbles begin at 6.5 cm. Hg	

The rabbit inoculated from the herpes filtrate remained negative, indicating that the herpes virus in the condition in which it existed in the suspension was larger than the collargol.

*Experiments Controlling the Possible Effect of Opposite Electrical Charges on Substances to Be Filtered and on Filter Membrane, Respectively.*

The following three experiments were carried out for the purpose of controlling errors in the results of filtration which could conceivably have occurred if the isoelectric point of the particles to be filtered should happen to be on the alkaline side of pH 7.2, at which all our previous experiments were done. Olitsky and Boéz stated that the isoelectric point of the virus of foot-and-mouth disease lay at about pH 8. It seemed to us likely that if foot-and-mouth disease virus were isoelectric at this reaction, the same might be true of the viruses with which we have been dealing. Were this the case, then of course casein and the other proteins, the isoelectric points of which are in the neighbourhood of pH 5, would be negatively charged at pH 7.2, and thus easily pass through a negatively charged membrane, while the viruses at pH 7.2 would be positively charged and might be held up by conditions entirely independent of size.

For this reason, in the following three experiments Rous sarcoma virus, herpes virus and staphylococcus bacteriophage were all compared by filtration, in each case through four parts of the same membrane, and both the casein and the respective substance adjusted to 7.2 in one segment and to 8.6 in another.

The virus used was a 2 per cent suspension of a freshly prepared triturate of Rous sarcoma filtered through a sand filter. The casein solution was prepared with the purified casein furnished us by Dr. Edwin Cohn.

*Membrane Q 4.*—1 per cent— $\frac{1}{4}$  min.—6—4 min.—30 min. 90 per cent. Divided into four parts.

	Water at 2 cm. Hg 5 min.	Filtration at 2 cm. Hg 7 min.
	cc.	
Q 4a	1.25	Casein pH 7.2, 0.75 cc. + came through
Q 4b	1.25	Casein pH 8.6, 0.75 cc. + + + came through
Q 4c	1.25	Rous sarcoma virus pH 7.2, 1.5 cc.
Q 4d	1.25	Rous sarcoma virus pH 8.6, 1.5 cc.

Chicks inoculated from Q 4c and Q 4d remained negative. Control chicks inoculated with the paper pulp sand filtrate before collodion filtration were positive in 2 weeks.

*Membrane Q 5.*—1 per cent— $\frac{1}{2}$  min.—6–4 min.—30 min. 90 per cent—divided into four parts.

The herpes virus consisted of a 7 day glycerolated brain of an herpetic rabbit; 1 gm. ground in sand and suspended in 30 cc. of Ringer's solution; centrifugalized at high speed until the supernatant fluid was clear. Casein as in the preceding experiment.

	Water at 2 cm. Hg 5 min.	Filtration 2 cm. Hg 7 min.	Water at 2 cm. Hg 8 min.
	cc.		cc.
Q 5a	2.75	Casein at pH 7.2, 0.75 cc. + came through	0.5
Q 5b	2.75	Casein at pH 8.6, 0.75 cc. ++ came through	1.0
Q 5c	2.5	Herpes virus at pH 7.2, 0.5 cc.	1.0
Q 5d	2.5	Herpes virus at pH 8.6, 1.0 cc.	1.0

Rabbits were inoculated from filtrates of Q 5c and Q 5d and controls with unfiltered supernatant fluid of centrifugalized virus taken from material left on membrane. The results indicated that the virus had not come through.

#### *Comparison of Bacteriophage with Casein.*

Staphylococcus bacteriophage consisting of a Berkefeld filtrate of a cleared culture.

*Membrane R 1.*—1 per cent— $\frac{1}{2}$  min.—6–4 min.—30 min. 90 per cent.

	Water at 2 cm. Hg 6 min.	Filtration 2 cm. Hg 8 min.	Water at 2 cm. Hg 6 min.
	cc.		cc.
R 1a	1.75	Casein pH 7.2, 0.75 cc. + came through	0.25
R 1b	1.5	Casein pH 8.6, 0.75 cc. ++ came through	0.25
R 1c	1.5	Staphylococcus bacteriophage pH 7.2, 1 cc.	0.5
R 1d	1.5	Staphylococcus bacteriophage pH 8.6, 1 cc.	0.5

The bacteriophage was tested in the following way:

*Tube 1.*—1 cc. 7 hour staphylococcus culture + 0.1 cc. unfiltered bacteriophage at pH 7.2 = complete clearing up overnight.

*Tube 2.*—1 cc. 7 hour staphylococcus culture + 0.1 cc. filtered bacteriophage at pH 7.2 = negative.

*Tube 3.*—1 cc. 7 hour staphylococcus culture + 0.1 cc. unfiltered bacteriophage at pH 8.6 = complete clearing.

*Tube 4.*—1 cc. 7 hour staphylococcus culture + 0.1 cc. filtered bacteriophage at pH 8.6 = negative.

In the last experiment tabulated we used collargol as a colloidal suspension composed presumably of particles larger than any of the proteins used. We had found that the bacteriophage and the viruses were all held up by membranes which allowed the three proteins to pass through them. Curiously enough, although our calculations from the molecular weight—taking the density as 1 — indicated that the casein molecules should have a diameter of approximately  $8.5\text{ m}\mu$ , we found by actual filtration that casein was larger than collargol, which is supposed to have a minimum diameter of  $20\text{ m}\mu$ . Moreover, whatever the pH, casein solutions are always very slightly opalescent, a fact which should indicate that many of the particles in suspension cannot be much smaller than half a wave-length of light. It must be assumed, therefore, that casein is present either in a condition of considerable swelling or as aggregates. This is a matter that we are hardly competent to discuss in detail, but we offer the observed facts in the interests of completeness.

### III.

In attempting to produce membranes of increasing permeability in order eventually to obtain filters that would allow the bacteriophage and the two varieties of virus to pass through them, we found that the alcohol-ether method could not be satisfactorily used because of the friability of membranes containing less than 1 to  $1\frac{1}{2}$  per cent of collodion. We therefore turned to the method advised by Bechhold, Schoep and others.

This consists of impregnating filter paper with solutions of collodion in glacial acetic acid. The permeability of the membrane is entirely determined by the percentage of the collodion solution. The membranes are hung into a bath of acetic collodion in a vacuum chamber, the collodion being run into the bath after preliminary exhaustion, and after the air has been removed from the pores of the paper, atmospheric pressure forces the collodion solution thoroughly into the interstices of the paper. The filter paper is then allowed to drip and is finally soaked in running water for 24 hours.

Such membranes were employed in the same type of filter and in virtually the same manner as were the alcohol-ether films.

For comparative filtrations with these membranes we used, in addition to the substances to be tested, casein, collargol and freshly prepared arsenic sulfide.<sup>2</sup>

The following table shows that 4 per cent acetic-collodion lets through crystallized egg albumen, but holds back serum albumen and the larger particles of collargol and arsenic.

*4 Per Cent Acetic-Collodion Membrane.—*

Mem- brane	H <sub>2</sub> O, 15 min. 8 cm. Hg		15 min. 8 cm. Hg
	cc.		cc.
1	1.0	Egg albumen ++	0.5
2	0.75	Horse serum albumen negative	0.5
3	0.80	Collargol negative	1.0
4	1.25	As <sub>2</sub> S <sub>3</sub> negative	1.5

In the next experiment a more permeable membrane was used.

*2 Per Cent Acetic-Collodion Membrane.—*

Mem- brane	H <sub>2</sub> O, 10 min. 2 cm. Hg	Filtration 10 min. 2 cm. Hg	H <sub>2</sub> O, 10 min. 2 cm. Hg
	cc.		cc.
1	2.5	Collargol 1.75 cc., ++ bright yellow	
2	2.5	As <sub>2</sub> S <sub>3</sub> 1.50 cc. negative	0.75
3	2.0	Phage 0.75 cc. +	
4	2.5	Rous sarcoma 1.0 cc. negative. Control positive	0.75

This 2 per cent acetic-collodion membrane let through collargol and the bacteriophage, but held back the Rous sarcoma virus and the arsenic suspension. While

<sup>2</sup> The collargol suspension was made by dissolving 0.2 per cent commercial collargol both in distilled water and in salt solution, centrifuging for 2 to 3 hours at approximately 2,000 R.P.M. and using the upper layers of the fluid. The arsenic trisulfide was made by passing hydrogen sulfide through a solution of As<sub>2</sub>O<sub>3</sub> in water. The arsenious acid solution was saturated by boiling, was cooled to room temperature, filtered and diluted with 3 volumes of water. This is the method

the subsequent water tests on two of these membranes showed that there had been no gross leakage—indeed, a considerable obstruction,—the delicacy with which these experiments must be done, is indicated by the fact that Membranes 1 and 3 ruptured when the suction was increased to 4 and 5 cm. Hg.

In the next experiment herpes virus was compared with casein and collargol.

*2 Per Cent Acetic-Collodion Membrane.—*

Mem- brane	H <sub>2</sub> O, 5 min., 2 cm. Hg	Filtration	H <sub>2</sub> O, 5 min. 2 cm. Hg
	cc.		cc.
a	2.5	Herpes 2 per cent 0.75 cc.	0.75
b	2.5	Casein 1 cc. acid — negative, precipitate —++	0.75
c	2.5	Collargol 1.25 cc. +++ bright yellow	1.35

The control rabbit inoculated with the material over the filter membrane died in 5 days. The test rabbit inoculated with the filtered virus died with a typical syndrome of herpes at about the same time. Fresh herpes brain from a rabbit which had just died was used in the preparation of this virus, and we believe that this may have something to do with the successful filtration.

A still more permeable membrane was made and used to compare collargol, staphylococcus bacteriophage, Rous sarcoma virus and arsenic suspension.

*1.5 Per Cent Acetic-Collodion Membrane.—*

Mem- brane	H <sub>2</sub> O, 5 min., 2 cm. Hg	5 min., 2 cm. Hg	H <sub>2</sub> O, 5 min. 2 cm. Hg
	cc.		cc.
a	2.5	Collargol 2.5 cc. ++	2.5
b	3.0	Phage 2.0 cc. +	2.75
c	3.0	Rous sarcoma 2.5 cc.	2.0
d	2.5	As <sub>2</sub> S <sub>3</sub> 1.75 cc. negative	1.80

The chick inoculated with the filtrate of the Rous virus on the left breast, with a control inoculation of the unfiltered virus on the right breast, began to show definite tumors on both sides within 10 days. This experiment was repeated with another set of membranes, with entirely comparable results.

Combining this with previous experiments, it would be apparent that the Rous sarcoma virus was larger than collargol and smaller than colloidal arsenic.

described by Wolfgang Ostwald, Practical colloid chemistry, New York, 4th edition, 1924, 6.

*1.5 Per Cent Acetic-Collodion Membrane.—*

Mem- brane	H <sub>2</sub> O pH 7.2 5 min. at 3 cm. Hg	Filtration 10 min. at 4 cm. Hg	H <sub>2</sub> O pH 7.2 5 min. 3 cm. Hg
	cc.		cc.
a	2	Collargol 3 cc. +++	1.5
b	2.5	As <sub>2</sub> S <sub>3</sub> 4 cc. negative	1.0
c	2.5	Herpes* (0.25 per cent) 1 cc.	0.75

\* Control died typically in 5 days, filtrate rabbit on 8th day.

In the above experiment herpes virus apparently came through the membrane which held back colloidal arsenic. The final water tests show that no accidental leakage or other irregularity was responsible for the result.

## DISCUSSION.

The determination of size by methods of ultrafiltration is of course subject to many possibilities of error and cannot be relied upon except in defining relatively gross ranges of magnitude. We believe that the technique developed by us avoids all the obvious errors and is subject to simple control in regard to the detection of leakages, and injuries to the filter membranes in the course of the experiments. The practice of measuring the flow of water in a given time under constant pressure before and after filtration of various substances not only insures against accidents of the kind mentioned, but supplies interesting information in regard to the degree to which a membrane has been obstructed by the substance filtered. The method of *making membranes in sheets and dividing them into separate parts* makes it possible to filter several substances simultaneously under equivalent conditions, and the accuracy with which identical conditions can be assumed to prevail is further controlled by attaching all the different segments to the same source of negative pressure and determining the flow of water per unit time both before and after the filtration.

The alcohol-ether method of making membranes on rotating tubes, especially when reenforced by sheets of Japan paper, furnishes a delicate and very elastic method of varying permeability, but these membranes cannot be made sufficiently permeable to allow anything larger than collargol to pass through them without becoming too

soft and friable for use. More permeable membranes of sufficient strength can be made by the Bechhold method with collodion dissolved in glacial acetic acid, and this was therefore the method that we employed in the final stages of our experiments.

For determination of the sizes of the pores of our membranes we hoped to be able to use the method of Bechhold, which depends upon the measurement of the pressure necessary to force air through the filter under water. A formula has been derived by him from such measurements which has also been applied by Stuart Mudd to the measurement of the pores of Berkefeld filters. We found, however, that the calculated sizes of the pores in our collodion membranes were entirely inconsistent with the probable sizes indicated by permeability for various substances, a discrepancy that we attribute to the fact that each additional cm. of pressure upon the soft filter membranes used by us considerably bulged them in a manner which inevitably modified the pore sizes, and pressures beyond 4 and 6 cm. of mercury often completely disrupted these filters. This rendered it essential to control our membranes by the flow of water under similar pressures before and after each experiment. It will be noticed that we hardly ever used more than 2 cm. of mercury for our filtrations. Poiseuille's formula, which is based upon measurements of the pressure necessary to force water through a capillary, and has been modified by Bechhold to apply to filters, was also found inapplicable in our studies.

#### CONCLUSIONS.

We are submitting this series of experiments as observed facts, realizing that there are so many uncertainties in this form of indirect observation that great caution must be exercised in drawing conclusions of any kind. The most serious of the possible errors involved is that the active substances which we have studied, the enzyme—the bacteriophage—and the several varieties of virus, may not be free in our suspensions, but are adsorbed to larger particles. The peculiar difficulties encountered in filtering herpes virus particularly suggest a source of error of this kind, and if we are right in assuming the intracellular position of this virus in the nervous tissue, it is more



than likely that most of the virus obtained in suspension may be closely associated with protein particles derived from the cells. Keeping all this in mind, we may, nevertheless, derive a certain amount of information from our experiments as follows:

1. The order of magnitudes of the pure proteins with which we have worked,—namely, crystallized egg albumen, crystallized serum albumen and purified casein,—follows the order of molecular weights of these substances as determined by Cohn. As far as casein is concerned, the size indicated by filtration in comparison with collargol is far greater than it should be by calculations which take a molecular weight of 192,000 as the point of departure. While one cannot be sure of the reason for this, there are many possible explanations such as considerable swelling of the casein particles, aggregation of molecules and the fact that casein is not at its isoelectric point under the conditions of filtration and surely present as a salt.

2. Trypsin, even in the certainly very impure condition in which we employed it, is but very slightly larger than serum albumen and distinctly smaller than casein. In its pure form it may well be much smaller even than our filtrations indicate, but certainly not larger. This relatively small size of trypsin may have considerable bearing upon the question of whether or not the lytic agents spoken of as "bacteriophage" are substances of the nature of enzymes, or whether they are more comparable to the filtrable virus, as supposed by d'Hérelle.

3. Herpes virus, the Rous chicken sarcoma and a staphylococcus bacteriophage were all subjected to filtration at pH 7.2 and at hydrogen ion concentrations higher than 8, which is given by Olitsky and Boëz as the isoelectric point of foot-and-mouth disease, but failed to pass membranes which, at the same pressures, were permeable for casein and collargol. The bacteriophage and the Rous sarcoma with considerable regularity passed through membranes which held back colloidal arsenic trisulfide. We have cited only a few of the experiments which were actually done, every one of the tests tabulated being merely representative of a number of others that were omitted for economy of space. The herpes virus we have had greater difficulty in filtering. We cite one experiment with a 2 per cent acetic-collodion membrane and another with a 1.5 per cent

membrane through which the herpes virus passed, the membrane being so controlled that gross leakage could be excluded. We believe that the difficulty here is very largely due to the fact that in preparing the herpes virus for filtration it cannot be separated from considerable amounts of brain material, from which, perhaps, it is not easily dissociated. This would be natural if the herpes virus were intracellularly located, as we believe it to be. This experiment and similar ones, however, incline us to believe that the herpes virus is not far different from the Rous sarcoma virus and the bacteriophage, as far as filtration through membranes is concerned. It certainly is not smaller than either of these substances and probably, as we judge from a few experiments carried out at higher pressures, is not much larger.

It may be assumed, therefore, that in the form in which we were able to procure the bacteriophage and the two varieties of virus investigated by us, they were of a magnitude larger than casein and collargol and smaller than colloidal arsenic. The weak point in drawing our conclusions is the fact that we were not in a position to measure for ourselves with any accuracy the actual sizes of collargol and arsenic trisulfide particles. Accepting the general views of Bechhold and others, however, our experiments would define the sizes of the separticular substances as larger than  $20\text{ m}\mu$  and probably smaller than  $100\text{ m}\mu$ .

The order of magnitudes of the substances measured by us would then be as follows:

Crystallized egg albumen  
Crystallized serum albumen  
Trypsin  
Collargol  
Casein  
Bacteriophage, Rous sarcoma virus, herpes virus  
Arsenic trisulfide

Our experiments show little agreement with the work of Levaditi and Nicolau and of Levaditi, Nicolau and Galloway. In their recent filtration tests of foot-and-mouth disease this virus is reported by them as passing through membranes that held back trypsin, indicating a size much smaller than any of the viruses measured by us.

Our results, on the other hand, are in actual measurements comparable to those of Olitsky and Boëz, not only in the fact that the viruses with which we worked correspond approximately to the size determined by them for foot-and-mouth disease, but that the percentage of collodion in membranes permeable for virus and impermeable for colloidal arsenic corresponds almost exactly to our own. This gives us confidence that the technique developed may be more easily standardized than we at first believed and that the method of ultrafiltration, owing to the great ease with which membranes of relatively standard size may be made, may have valuable applications in the investigation of bacteriological and immunological problems.

## BIBLIOGRAPHY.

1. Rivers, T. M., *J. Bact.*, 1927, xiii, 16.
2. Mudd, S., and Warren, S., *J. Bact.*, 1923, viii, 447; Mudd, S., *J. Bact.*, 1923, viii, 459.
3. Bechhold, H., *Z. physik. Chem.*, 1907, lx, 257; 1908, lxiv, 328.
4. Levaditi, C., and Nicolau, S., *Compt. rend. Acad.*, 1923, clxxvi, 717; Levaditi, C., Nicolau, S., and Galloway, I.-A., *Compt. rend. Acad.*, 1926, clxxxii, 247.
5. Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, 685.
6. Brown, W., *Biochem. J.*, 1915, ix, 594.
7. Nelson, J. M., and Morgan, D. P., Jr., *J. Biol. Chem.*, 1923, lviii, 305.
8. Hitchcock, D. I., *J. Gen. Physiol.*, 1925, viii, 61.
9. Schoep, A., *Bull. Soc. chim. Belge*, 1910, xxiv, 354.
10. Cohn, E. J., *Physiol. Rev.*, 1925, v, 349.

# NATURE OF THE TOXIC MOIETY OF STREPTOCOCCUS SCARLATINÆ.\*

By CHARLES W. DUVAL, M.D., AND R. J. HIBBARD, M.D.

(From the Department of Pathology of Tulane University, New Orleans.)

(Received for publication, May 16, 1927.)

Scarlet fever is now generally regarded as an infection caused by hemolytic streptococci, but there is much that is imperfectly understood concerning the nature of the toxemia and the natural and artificially induced immunity. There are those who, recognizing the streptococcus as the primary excitant of the disease, believe that the specific poison is a soluble toxin comparable to that of diphtheria, and others hold that it is endotoxic in kind.

Dick and Dick<sup>1</sup> regard the active principle as a soluble toxin elaborated by the streptococcus during its *in vitro* growth activity, and base their belief upon the fact that it is neutralized by immune serum. Dochez<sup>2</sup> apparently could not procure from *in vitro* culture filtrate a toxic substance which would induce an anti-toxic body when injected into animals. On the other hand the results of his ingenious *in vivo* experiments with living streptococcal impregnated agar lead him to consider that a soluble toxin might be formed in the living animal. Rosenow<sup>3</sup> claims to have obtained thermolabile and thermostable toxic moieties of the scarlatinal streptococcus since both the "washed bacterial bodies" and the heat-killed streptococcal cell remove the neutralizing principle of the homologous immune serum; he believes that the "soluble toxin" and endotoxin are one and the same thing. Eagles<sup>4</sup> reports that the culture filtrates from a widely distributed group of hemolytic streptococci give rise to a skin reaction in the human which is indistinguishable from the Dick reaction, stating that the culture filtrates of all hemolytic strains, regardless of their source, are neutralized by scarlatinal horse

---

\* Aided by a grant from the David Trautman Schwartz Research Fund of Tulane University.

<sup>1</sup> Dick, G. F., and Dick, G. H., *J. Am. Med. Assn.*, 1924, lxxxii, 301.

<sup>2</sup> Dochez, A. R., *J. Am. Med. Assn.*, 1924, lxxxii, 542.

<sup>3</sup> Rosenow, E. C., *J. Infect. Dis.*, 1925, xxxvi, 525.

<sup>4</sup> Eagles, G. H., *Brit. J. Exp. Path.*, 1926, vii, 286.

serum. Williams<sup>5</sup> states that streptococci from cases of bronchitis and osteomyelitis yield toxic filtrates which are neutralized by convalescent scarlet fever serum and by immune horse serum. Birkhaug<sup>6</sup> using crysipelas strains determined that the toxic filtrates produce a skin reaction similar to the scarlatinal toxin; while Duval and Hibbard<sup>7</sup> were unable to obtain toxic effects in rabbits with the culture filtrate, yet definite symptoms followed the injection of streptococcal lysate.

With a view of obtaining further information on the nature of the scarlatinal toxin several lines of investigation were undertaken. One of these was a comparative study of the cutaneous reaction in the human non-immune to the *in vitro* produced toxin (Dick's culture filtrate) and the *in vivo* prepared streptococcal endotoxin (Duval and Hibbard's culture lysate); another, to determine by the intradermal test the neutralizing effect of immune serum upon the specific "filtrate" and "lysate" respectively, while a third series of experiments was conducted to determine the infectivity of scarlet fever streptococci for the dog, and to compare the toxic effects produced in this animal with injections of culture filtrate and lysate. A study was also made of the antibody content of sera from rabbits immunized separately against filtrate and lysate, particularly with respect to lytic and neutralizing antibodies.

#### *Materials and Animals Employed.*

The bacterial lysate employed in our studies was prepared from the Dick "Harrison" strain of *Streptococcus scarlatinæ*. Heavy growth of the culture was first obtained upon nutrient sheep serum agar which had been slanted in quart size sterile whiskey flasks and incubated for 3 days at 37°C. The surface growth of each flask was then washed down and suspended in 50 mls of sterile normal salt solution. The entire bacterial suspension of one or more flasks was now injected into the peritoneal cavity of rabbits which had been previously immunized against the homologous culture. 2 to 3 hours after the introduction of the living streptococci, the animal was sacrificed and the peritoneal fluid was collected and passed through an N Berkefeld filter. The resulting filtrate which contains the product of the *in vivo* dissolved cocci constitutes what is herein called lysate. This was then standardized, by means of the method described by Dick and Dick for the

---

<sup>5</sup> Williams, A. W., *Am. J. Pub. Health*, 1925, xv, 129.

<sup>6</sup> Birkhaug, K. E., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 248.

<sup>7</sup> Duval, C. W., and Hibbard, R. J., *J. Exp. Med.*, 1926, xliv, 567.

standardization of culture filtrate. A skin test dose was considered a standard unit of lysate, and is defined as that amount which will give a positive skin reaction in the non-immune to scarlatina and a negative reaction in the immune. Since susceptibility and resistance to scarlet fever are relative terms it is obvious that no fixed amount of lysate will satisfy the definition given for a standard unit, even if the cutaneous reaction was the only factor in determining susceptibility.

The culture filtrate (Dick's scarlatinal toxin) used was from two different lots; one was kindly sent us by Dr. Dick (0.1 cc. of a 1:250 dilution equaled a skin unit), the other was prepared in our own laboratory.

The scarlatinal immune sera employed were of three different lots; one, Eli Lilly's scarlet fever antitoxin (obtained in the open market) and the others, from (1) rabbits immunized with culture lysate and (2) rabbits immunized with culture filtrate of *Streptococcus scarlatinæ*.

As the dog proved to be highly susceptible of infection with *Streptococcus scarlatinæ*, this animal was utilized instead of the rabbit for certain of the experiments herein reported. Normal young dogs commonly develop a generalized infection following the injection of living culture, and succumb in 3 to 5 days from an acute hemorrhagic nephritis which is the most prominent feature of the induced toxemia. The killed culture and culture lysate are also highly toxic for this animal. All dogs were kept under observation for a period of 2 weeks prior to inoculation, during which time daily analysis of the urine was made and the blood chemistry determined. Only dogs with normal kidney function were employed for experimentation. The preliminary study of the blood and urine was carried out by our colleague, Professor Denis, head of the Department of Bio-Chemistry.

Rabbits only were employed for immunization. The immune sera of these animals were utilized to determine the kinds of antibodies experimentally induced with culture filtrate and lysate antigens respectively. Furthermore the sera were used as indicators in the determination of the relative toxic strengths of lysate and filtrate.

#### EXPERIMENTAL.

*Experiment 1.*—In order to compare the reactivity of the human skin to lysate and filtrate, and to determine the relative strengths of these two products 80 volunteer medical students were inoculated intradermally with 0.1 cc. quantities of varying dilutions of the respective materials. Saline and bouillon in similar doses were used as controls. Three different strengths of each product were prepared and the injections made at intervals of 3 inches apart along the flexor surface of the forearm, the right was used for lysate and the left for filtrate. The lysate dilutions were 1:2000, 1:1000 and 1:500 while the filtrate was utilized in strengths of 1:250, 1:100 and the undiluted. Readings were made at 24 and 48 hour intervals and all tests were considered negative if after this period no definite area of redness occurred about the inoculation site.

The remarkable feature of the culture lysate reaction was its intensity and large involvement of skin area, while the reaction to culture filtrate was seldom more than a mild erythematous blush for a relatively small area of skin. As a rule the reaction to culture lysate was clear-cut, distinctly elevated, dark red in color and usually 3 cm. in extent.

Presumably the toxicity of lysate is greater because it contains not only a larger quantity of poison but is the lysin-separated pure toxic product of a large number of streptococcal cells. On the other hand Dick's culture filtrate is simply the autolyzed product of the dead coccal cells. It would seem that lysate is capable *per se* of directly injuring the tissues while filtrate has not this property until altered by some lytic host factor. However, this difference in the reactive

TABLE I.  
*Comparative Percentages of Reactions to Lysate and Filtrate.*

Number persons tested	Positive skin reaction to filtrate (Dick's toxin). Dose, 0.1 cc. of the following Dilutions of filtrate			Positive skin reaction to lysate (Duval and Hibbard toxin). Dose, 0.1 cc. of the following Dilutions of lysate		
	Undiluted	1:100	1:250	1:500	1:1000	1:2000
80	54 (67.5%)	22 (27.5%)	14 (17.5%)	18 (22.5%)	17 (21.5%)	13 (16.5%)

power of lysate and filtrate is no proof that the toxic principle of *Streptococcus scarlatinæ* is endotoxic though it may be inferred that such is the case.

Table I gives the results and comparative percentages of reactions. It is seen from the table that 67.5 per cent reacted to the undiluted, 27.5 per cent to the 1:100 and 17.5 per cent to the 1:250 dilution of culture filtrate. While 22.5 per cent reacted to the 1:500, 21.5 per cent to the 1:1000 and 16.5 per cent to the 1:2000 dilution of lysate. Since a 1:2000 dilution of lysate reacted in as high a percentage of cases as the 1:250 dilution of filtrate (Dick's skin unit) the comparative strengths of the two toxic products are indicated as 1 to 10. In other words lysate, at least for the batch employed, is ten times the strength of filtrate. This difference in the reactivity leaves no doubt

regarding the greater degree of toxicity for lysate as compared to filtrate.

*Experiment 2.*—To determine the amount of immune serum, in terms of skin units, necessary to neutralize a standard skin dose of scarlatinal lysate and to compare this with the amount required to neutralize a unit of culture filtrate, mixtures of the respective toxin-immune serum were prepared and injected intradermally. Three different lots of immune sera were employed: (1) Eli Lilly's globulin fraction of scarlatinal horse serum, (2) immune rabbit serum produced with Dick's culture filtrate and (3) immune rabbit serum produced with our culture lysate. Dilutions of 0.1 cc., 0.2 cc., 0.3 cc. were made in sterile normal saline of the various sera. To separate series of these dilutions were added and thoroughly mixed, one skin unit of lysate and filtrate respectively. The mixtures were allowed to stand in diffuse light for 1 hour before 0.1 cc. quantities were injected.

Fifteen medical students in whom the culture filtrate and culture lysate reactions were positive, volunteered for the test. These were divided into three groups of five each, one group for each immune serum to be tested. The toxin-antitoxin mixtures were injected into the skin of the forearm at different sites and approximately 3 inches apart. The first group received the Eli Lilly serum-toxin mixtures, the second group the "lysate" immune rabbit serum-toxin mixtures and the third group the "filtrate" immune rabbit serum-toxin mixtures. Table II shows the results obtained in this experiment.

Dilutions of 1:250 scarlatinal horse serum neutralize completely one unit quantities of culture filtrate and culture lysate. Dilutions of 1:250 of "lysate" immune rabbit serum neutralize one unit of culture filtrate, while a dilution of 1:65 of "lysate" immune rabbit serum neutralizes a unit of culture lysate. The "filtrate" immune rabbit serum in dilution 1:65 neutralizes a unit of filtrate but fails to neutralize a unit of lysate. These results prove that the immune horse serum employed was of greater antitoxic strength than the sera of rabbits which were immunized against lysate and filtrate. There is also demonstrated the fact that lysate as compared to filtrate contained relatively more toxin.

*Experiment 3.*—Since scarlet fever is known to occur in a certain percentage of persons in whom susceptibility is not indicated by the skin test unit of Dick and Dick, the following experiment was carried out to show that a negative reaction is at best merely an index to a relatively small range of immunity. 80 volunteers were first skin-tested with three different strengths of Dick's culture filtrate, namely the undiluted, 1:100 and 1:250. Twenty-six of the number gave no skin reaction to one unit of Dick's toxin. These non-susceptibles or immunes, as



TABLE II.

*Neutralization of Scarlatinal Toxin (Lysate and Filtrate) with Immune Sera as Determined by Intradermal Injections of the Mixtures.*

Persons tested	Positive skin test to 0.1 cc. dosage of dilutions		Filtrate plus serum (lysate immune rabbit) Left arm Serum dilutions			Filtrate plus serum (Eli Lilly) Right arm Serum dilutions		
	Filtrate	Lysate						
	1:250	1:1000	1:250	1:125	1:65	1:250	1:125	1:65
1	+	+	—	—	—	—	—	—
2	+	+	—	—	—	—	—	—
3	+	+	—	—	—	—	—	—
4	+	+	—	—	—	—	—	—
5	+	+	—	—	—	—	—	—
	Positive skin test to 0.1 cc. dosage of dilutions		Lysate plus serum (filtrate immune rabbit) Left arm Serum dilutions			Lysate plus serum (Eli Lilly) Right arm Serum dilutions		
	Filtrate	Lysate						
	1:250	1:1000	1:250	1:125	1:65	1:250	1:125	1:65
6	+	+	+	+	+	—	—	—
7	+	+	+	+	+	—	—	—
8	+	+	+	+	+	—	—	—
9	+	+	+	+	+	—	—	—
10	+	+	+	+	+	—	—	—
	Positive skin test to 0.1 cc. dosage of dilutions		Lysate plus serum (lysate immune rabbit) Left arm Serum dilutions			Lysate plus serum (filtrate immune rabbit) Right arm Serum dilutions		
	Filtrate	Lysate						
	1:250	1:1000	1:250	1:125	1:65	1:250	1:125	1:65
11	+	+	+	—	—	+	+	+
12	+	+	+	+	—	+	+	+
13	+	+	+	+	—	+	+	+
14	+	+	+	—	—	+	+	+
15	+	+	+	+	—	+	+	+

+ equals skin reaction and no neutralization.

— equals no skin reaction and neutralization.

indicated by a negative cutaneous reaction, were then skin-tested with varying dilutions of culture lysate (1:500, 1:1000, 1:2000 respectively). Thirteen of the twenty-six gave a strongly positive reaction to the 1:2000 dilution of lysate, seventeen to the 1:1000 and eighteen to the 1:500. Eight cases failed to react.

Thus it was shown that a high percentage of negatives to the Dick tests react to lysate, from which fact it may be inferred that one skin

TABLE III.

*Intradermal Reaction to Different Dilutions of Lysate and Filtrate in Persons Susceptible to Scarlet Fever.*

	Scarlatinal lysate (dose 0.1 cc.)			Scarlatinal filtrate (dose 0.1 cc.)		
	Dilutions			Dilutions		
	1:500	1:1000	1:2000	Undiluted	1:100	1:250
1	+++	+++	++	++	+	+
2	+++	+++	+	++	+	-
3	+++	++	-	++	+	+
4	+++	+++	++	++	+	+
5	+	-	-	-	-	-
6	+++	+++	+	++	+	-
7	++	+	-	+	+	-
8	+++	++	+	++	+	+
9	+++	++	-	++	+	+
10	++	+	-	+	-	-
11	+	-	-	-	-	-
12	+++	++	+	-	+	-
13	+++	++	-	++	+	-
14	++	+	-	++	+	+
15	++	+	-	+	-	-
16	+++	++	+	++	+	+

Degrees of reaction are indicated by + + +, + +, +.

The minus sign (-) indicates a negative reaction.

unit of culture filtrate contains relatively too little toxic substance in excess of what may be neutralized by natural antibodies present in the human host. It is reasonable to suppose that the antibodies persist in persons who have had at some previous time a focal streptococcal infection.

*Experiment 4.*—Since an animal more readily susceptible to infection with scarlatinal culture than those previously used (rabbit, guinea pig and mouse) might serve as a means of determining the nature of the toxic principle of *Streptococcus scarlatinæ*, a series of tests was carried out upon the dog. It was found that small doses of viable culture introduced subcutaneously, intravenously or intraperitoneally resulted in a generalized infection and often death of the animal in from 3 to 5 days. It was also found that the dog is highly susceptible to the toxic product of the *in vivo* killed and dissolved scarlatinal streptococcus culture, and little if at all susceptible to the culture filtrate of Dick and Dick. Previous to inoculation all dogs were examined daily for the kidney condition over a period of 10 days. The animals used were those with normal kidney function.

(*Infectivity of Dog.*) Six full grown healthy dogs were inoculated with living cultures of scarlatinal streptococcus (Dick's Harrison strain). Two animals received the injection intravenously, two subcutaneously and two intraperitoneally. In each instance the dosage was 10 mls of the surface growth from slanted sheep serum agar which had been washed off and suspended in 50 mls of sterile normal saline. All the animals developed promptly a generalized infection and died in 2 to 5 days following the inoculation. Daily urine examinations showed pus, blood, albumin and casts. At autopsy there were the usual gross signs of sepsis, and in addition a markedly acute hemorrhagic nephritis. In one dog the kidneys were studded with streptococcal abscesses. Pure culture of the streptococcus was recovered from the lesions.

It is evident from the results obtained that the dog is the animal of choice because of the ease with which infection is induced independently of the route of administration. It is noteworthy that the dog is highly susceptible compared to other animals previously used. The constant occurrence of acute glomerulonephritis is also of special interest.

*Experiment 5. (Toxic Effects of Lysate and Filtrate upon the Dog.)*—Four young healthy dogs were inoculated intravenously with 10 mls each of filtered lysate which had been prepared *in vivo* in the manner elsewhere described. Four of the normal dogs received intravenously 10 mls of culture filtrate (Dick's toxic broth). The animals receiving lysate developed within 4 hours symptoms of toxemia, and 24 hours later were extremely ill. The urine macroscopically was bloody and analysis showed quantities of albumin, granular casts, bile and blood. Two of the animals died on the 4th day following the inoculation. The others survived, and though apparently well have continued to show albumin and casts in the urine. The four dogs that received culture filtrate did not show at any time signs of toxemia and have remained perfectly well.

The animals of this experiment reveal a marked difference in toxic effects for the two products employed. Undoubtedly the lysate is

much more toxic than filtrate though the identical quantities of each were injected. The experiment proves that large amounts of filtrate from cultures grown for 10 days in nutrient broth contain little toxin for the dog. This apparent difference in the reactionary property of lysate and filtrate indicates that the poison of *Streptococcus scarlatinæ* is intracellular. The toxin of filtrate is little in amount because it is derived from only the dead cocci of culture while that of lysate is the split product of large numbers of coccal bodies.

*Experiment 6. (Toxic Effect of Filtrate versus Killed Coccal Bodies.)*—Four young normal dogs were intravenously injected, two with the filtrate of a 3 day old culture and two with the residue of killed cocci from the same culture. The filtrate animals each received 15 mls which was one-half of the total amount of the Berkefeld filtered culture, and each of the other dogs was given one-half of the coccal residue (15 mls suspended in sterile normal saline). The dogs inoculated with filtrate appeared perfectly well throughout the period of observation. Daily examination of the urine and one blood chemistry analysis made on the 5th day revealed no abnormalities. The animals inoculated with coccal body residue showed severe toxic symptoms within 24 hours following the injection, one dying on the 3rd day and the other 2 days later of acute hemorrhagic nephritis. In one of the animals there was complete suppression of urine 2 days prior to death. The urine of both animals showed from the beginning of illness quantities of albumin, blood, bile and fine granular casts.

This experiment proves conclusively that the liquid medium of a 3 day old culture of scarlatinal streptococcus contains no poisonous substance for the dog while the coccal bodies are highly toxic for this animal, from which it may be inferred that the toxic principle is intracellular and not soluble in the exotoxin sense of the term. Apparently the toxin of culture filtrate is the product of autolyzed dead streptococci since the broth of a 3 day old growth, in which there are relatively few dead organisms, is non-toxic and that of older cultures is toxic because the culture now contains a larger number of dead cocci.

*Experiment 7. (Rabbit Immunization.)*—In order to determine whether there are differences in lysin and antitoxin content for scarlatinal immune sera produced with filtrate and lysate antigens, rabbits were separately immunized with these substances. Both antigens were prepared from Dick's Harrison strain of scarlatina. The animals were injected intravenously with gradually increasing doses at intervals of 3 days over a period of 6 weeks. 10 days after the last injection the animals were bled and the serum tested for lysin and neutralizing antibodies. The complement fixation method was used for the detection of lysin while toxin-

immune serum mixtures injected into the skin of human susceptibles was the method employed in the detection of neutralizing antibodies.

The serum of rabbit immunized against filtrate contained both lysin and antitoxin; however these were present only in small amounts. The serum from animal treated with lysate was rich in neutralizing antibody but no lytic substance was detected. These results are significant because in the instance of the animal treated with filtrate it may be assumed that the antigen was not only small in amount but of a whole protein nature, while with the animal immunized with lysate the antigen was truly a neutralizing antibody producer. The fact that the serum of rabbit immunized against lysate contains only antitoxin is evidence that the poison is the endotoxic split product of the streptococcal cell. On the other hand culture filtrate, it would seem, is the whole protein of the disintegrated cell and is an antigen substance which gives rise to a lytic immunity.

#### DISCUSSION.

The results of the various experiments and tests conducted leave little doubt as to the endotoxic nature of the scarlatinal poison. In our experience animal inoculation with either culture or the filtrate gives rise to an immunity which is more lytic than antitoxic. While neutralizing antibodies are induced with these antigens, bactericidal substances occur in far greater proportion in the immune serum. In consequence of this the toxic principle of scarlet fever is not comparable to a microbic exotoxin like that of diphtheria.

The filtrate of broth-grown cultures is relatively weak in toxin compared to corresponding amounts of dead coccal bodies or culture lysate, though the filtrate from older broth cultures is proportionately more toxic than that of younger cultures. For example, 15 mls of filtrate of a 3 day old broth culture fails to give rise to toxic effects in the susceptible animal while the same amount of filtrate from a 2 weeks old culture is toxic. Therefore it would seem that the toxin in culture filtrate is the product of the autolyzed dead streptococci and, like that of the coccal bodies, is bound up in the cytoplasm of the cell. On the other hand culture lysate (prepared in the abdominal cavity of the immune rabbit) induces only neutralizing antibody from which it may be inferred that the latter is the pure separated poison of the strepto-

coccal cell. This difference is shown by lysate antigen stimulating only the production of neutralizing immune substance, and coccal bodies and culture filtrate primarily producing lytic antibodies.

The neutralization of the toxic substance of culture filtrate with the homologous immune serum does not necessarily mean that the poison is an exotoxin or that the neutralizer, strictly speaking, is an antitoxin. Intracellular microbic poisons (endotoxin) stimulate the production of their corresponding neutralizers which properly designated are antientdotoxins. In our opinion it is clearly indicated that the toxic principle in scarlatina is intimately associated with the protein of the streptococcal cell, and only liberated *in vivo* by the action of a host lysin. The action of this lysin is of special interest as it appears to possess group cleavage properties, splitting equally as well certain other streptococcal antigens. For example the toxic principle of *Streptococcus viridans* and *erysipelatis* is liberated when these are introduced into the peritoneal cavity of the rabbit immunized against *Streptococcus scarlatinæ*. This behavior would suggest a close biological relationship between these respective organisms.

It is not known whether the toxin of the culture lysate is something that exists as an independent moiety within the bacterial cell or a new substance formed through chemical changes taking place concurrently with the disintegration of the cell. However, the experimental evidence is strong that it is the product of the specific action upon the bacterial cell by a host lysin. To what extent, if any, the host lysin enters into the lysate product is not known.

The relative ineffectiveness of an immune serum in the treatment of streptococcal infections as compared with the efficiency of antitoxic serum produced with true bacterial toxin has long been recognized. While antistreptococcal serum is effective in the treatment of scarlet fever and, as it would seem, to a far greater extent than immune sera for other streptococcal infections, it is not comparable in toxin-neutralizing power to antidiphtheritic and antitetanic sera. In the light of our experiments a more potent serum for scarlet fever and other streptococcal infections should be produced with antigens that are essentially stimulators of neutralizing antibodies. Such antigens are the *in vivo* prepared lysates which produce antitoxin in greater amount than is the case with living, killed culture or culture filtrate. A purely antitoxic serum will not increase the toxemia while

theoretically a lytic serum will, owing to the simultaneous cleavage *in vivo* of large numbers of streptococci in consequence of which there are liberated greater quantities of specific poison. We believe, however, that the present day antiscarlatinal serum though more lytic than antitoxic, contains sufficient neutralizing antibodies to neutralize the liberated toxin.

#### SUMMARY.

The cutaneous reaction demonstrates that the culture lysate of *Streptococcus scarlatinæ* is approximately ten time more potent in its toxic effect than is the culture filtrate since repeated and carefully controlled human skin tests show that 0.1 cc. of a 1:2000 dilution of lysate reacts equally as well as a similar dose of a 1:250 dilution of culture filtrate (Dick's standard skin unit).

Animal tests and the human intradermal reaction clearly reveal that the toxic principle of culture filtrate (Dick's toxin) and culture lysate (Duval-Hibbard endotoxin) are of the same nature, namely intracellular derivatives of the streptococcal cell.

The *in vivo* prepared lysate affords a more potent antigen for the production of an antiendotoxic serum than the living, killed or culture filtrate of *Streptococcus scarlatinæ*.

The inoculations into dogs of culture filtrate and of the "washed coccal bodies" yield strikingly different results. In those that receive filtrate no toxic effect is produced while in the ones injected with the washed coccal bodies a severe and often fatal toxemia results.

The dog is highly susceptible to infection with *Streptococcus scarlatinæ* and also readily affected by injections of the *in vivo* prepared lysate. Toxic effects are produced almost immediately following the intravenous injection of lysate and death usually occurs in 24 to 48 hours from an acute hemorrhagic nephritis. Daily urinary examination shows a high percentage of albumin, large numbers of fine granular casts and quantities of macroscopic blood. A study of the kidney sections reveals an extensive glomerulonephritis.

The work reported constitutes further evidence in support of our original contention that the poisonous substance of the scarlatinal streptococcus is derived from the bacterial cell set free through the dissolution of the germ plasm. The liberation of the poison *in vitro* occurs as the natural result of autolysis while *in vivo* it is produced through specific action of bacteriolysin.

# The Journal of General Physiology

Edited by

W. J. CROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

*The Journal of General Physiology* is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter.

*The Journal of General Physiology* is issued bimonthly, one volume of about 600 pages appearing in a year. Contributions should be sent to the editors of *The Journal of General Physiology*, The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y. The papers should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Papers will be printed as soon as possible after acceptance but not necessarily in the order in which they are received for publication. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (one volume) is \$5.00, payable in advance. Single copies cost \$1.00.

Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

## CONTENTS OF VOL. X, No. 6, JULY 20, 1927

- FALE, K. GEORGE, NOYES, HELEN MILLER, and LORBERBLATT, I. Studies on enzyme action-  
XLV. Lipase actions of the whole trout at different ages.
- NOYES, HELEN MILLER, LORBERBLATT, I., and FALK, K. GEORGE. Studies on enzyme action-  
XLVI. The ester-hydrolyzing actions of whole trout preparations under various conditions.
- RICHARDS, OSCAR W., and DAWSON, J. A. The analysis of the division rates of ciliates.
- STEVENS, KENNETH P. Studies on the amount of light emitted by mixtures of *Cypridina* luciferin and luciferase.
- HARVEY, E. NEWTON. On the quanta of light produced and the molecules of oxygen utilized during *Cypridina* luminescence.
- NORTHROP, JOHN H. The kinetics of osmosis.
- NORTHROP, JOHN H. The swelling of isoelectric gelatin in water. I. Equilibrium conditions.
- NORTHROP, JOHN H., and KUNITZ, M. The swelling of isoelectric gelatin in water. II. Kinetics.
- IRWIN, MARIAN. On the nature of the dye penetrating the vacuole of *Valonia* from solutions of methylene blue.
- GOWEN, JOHN W., and TOBEY, ELMER R. Udder size in relation to milk secretion.
- PERTZOFF, VLADIMIR. The effect of temperature upon some of the properties of casein.
- PERTZOFF, VLADIMIR. The effect of rennin upon casein. I. The solubility of paracasein in sodium hydroxide.

Published by

The Rockefeller Institute for Medical Research

Avenue A and 66th Street, New York, N. Y.





# LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

## I. EXPERIMENTAL PNEUMOCOCCAL MENINGITIS IN RABBITS.

By FRED W. STEWART, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 11 TO 15.

(Received for publication, May 31, 1927.)

Pneumococcal meningitis is not a common disease, and yet a certain number of cases appears each year in most larger hospitals. The disease is quite uniformly a fatal one. Whereas occasionally descriptions of recovery appear in the literature, one is frequently left with the impression that diagnosis was either incomplete or incorrect in such instances. To all intents and purposes, therefore, the disease may be considered as having a mortality of nearly 100 per cent. Consequently, any treatment, drastic as it may at first appear, which offers hope of benefit to even a small number of these unfortunate patients, may be worthy of consideration.

The problem of advancing the therapy of pneumococcal meningitis has interested this laboratory beginning with the study of Lamar (1). Lamar's work was done long before our far fuller knowledge of the pneumococci was obtained. His study was therefore somewhat incomplete from our present day point of view. Lamar was impressed by the fact that the pneumococcus was bile-soluble. It occurred to him, therefore, to attempt to influence the course of experimental pneumococcal meningitis by the intrathecal administration of mixtures of immune serum, sodium oleate, and boric acid. He had already noted (2) that pneumococci treated with sodium oleate became more subject to autolysis and that they became at the same time susceptible to serum lysis. This serum lysis tended to be incomplete with normal serum, but when immune serum was employed, lysis was complete, no multiplication of organisms took place in the test-tube mixture, and an inoculated animal was protected from infection. However, the soap action was inhibited in the presence of protein, and Lamar developed a method of minimizing this protein inhibition by adding to the serum-sodium oleate an appropriate quantity of boric acid. A number of monkeys was infected

directly in the lumbar subarachnoid space with a culture isolated from a human case of pneumococcal meningitis; the organism was not typed; it had only moderate mouse virulence, but produced in the monkey a fatal meningitis, closely resembling pneumococcal meningitis in man, save that the course of the experimental disease was more rapid. Lamar found that repeated administration of mixtures of sodium oleate, immune serum, and boric acid would arrest established infections and lead to enduring recoveries.

The value of this treatment may be determined only from its employment in human cases of the disease, and apparently little benefit followed its use (Kolmer (3)). Nor in our opinion have subsequent developments in therapy, such as Kolmer (3) and Kolmer and Idzumi (4) advocate, namely, saline lavage, or combinations of saline lavage with the administration of the antibody solution of Huntoon, or with various chemotherapeutic agents given much promise of beneficial results in man. Kolmer's results will be more fully discussed in a subsequent paper.

In view of all these facts, it was decided to reinvestigate pneumococcal meningitis. The first procedure consisted in a search for a satisfactory laboratory animal. Cats proved too resistant for our purposes; whereas with large doses of Type I pneumococci injected directly into the cisterna magna it was possible to produce acute leptomeningitis, the results were irregular and it was felt that it was not justifiable to draw conclusions from so resistant an animal. Attempts to produce the disease in guinea pigs were abandoned for similar reasons. The rabbit was at first reserved because of its high susceptibility, but was, nevertheless, next employed. In our experience the rabbit presents a difficult problem so far as highly virulent Type I pneumococcal infections are concerned in that, though extremely responsive to pneumococci, the production of meningeal disease in any way resembling that found in man was not readily attained.

If a normal rabbit receives Type I pneumococci in the cisterna magna, the development of septicemia is very rapid, and death is septicemic rather than meningeal. The cellular reactional processes common to human pneumococcal meningitis, as a rule, do not occur. Such, however, appears not to be the conclusion of Idzumi (5), for the latter reports vigorous leucocytic reactions due to Type I pneumococci in the rabbit meninges. Idzumi's pneumococci were apparently of low virulence, the mouse M.L.D. for his Type I being 0.001 cc. in 24 to 30 hours. It was employed in relatively enormous dosage—dosage at least 100 times as great as we have required to produce a fatal

septicemia without much meningeal reaction in a normal rabbit. In addition, Idzumi used a 24 hour culture, undoubtedly containing many damaged cocci. More recently pneumococcal meningitis has been studied in the rabbit by Untersteiner (6). The organism used was not typed, and we are therefore unable to analyze his results in terms of our own.

#### EXPERIMENTAL.

In studying pneumococcal meningitis of rabbits from the point of view of therapy, one is limited by several considerations: (1) the extreme susceptibility of the animal; (2) the invariable development of massive septicemia when virulent organisms are used—a septicemia which kills before the reactional process in the meninges develops to a sufficient extent to dignify it by the term “meningitis;” (3) the difficulty in lumbar punctures in the rabbit; (4) the limited working space for repeated injections into the cisterna magna resulting in frequent trauma; and (5) the rather soft consistency of the rabbit brain, rendering pressure deformation frequent.

It became apparent early that pneumococci injected into the rabbit's cisterna reached the blood stream in enormous numbers, beginning almost immediately and resulting in massive septicemia within so short a time as 5 hours after infection. Animals died within less than 24 hours, the anatomical cause of death being apparently intense edema and congestion of the lungs. Pneumococci could be demonstrated readily in the capillaries of all the large viscera (Figs. 1 and 2) and in blood smears. These findings led us to adopt methods of preventing the septicemia or of minimizing it to such an extent as to render it a negligible factor in the death of the rabbit. Naturally, recourse was had to partial immunization of the animals either passively, or actively. Rabbits were passively immunized by the intravenous administration, either just before cisternal infection, or a few hours previously. Usually 10 cc. of Type I antipneumococcus serum (therapeutic) was used. This serum was kindly supplied us by Dr. Augustus Wadsworth, of the Laboratory of the New York State Department of Health. Its potency was such that 0.2 cc. protected mice against simultaneous inoculation of 0.2 cc. of standard culture. Active immunity was established by one or two series of six intravenous

TABLE I.

No.	Type	Method of immunization	Infecting dose	Site of infection	Result of blood cultures					Duration of life	Post-mortem heart blood culture	Meningeal reaction
					1-1½ hrs.	2½-3 hrs.	5-5½ hrs.	24 hrs.	48 hrs.			
1	Actively immune	6 injections, 1 cc. vaccine, intravenously	0.5 cc.; 1:50, 18 hr. culture	Cistern	0	0	5	0	0	17 days	0	0
2	Control	None	0.5 cc.; 1:50, 18 hr. culture	"	25	370	∞			27 hrs.	+	+
3	Actively immune	6 injections, 1 cc. vaccine, intravenously	16,000,000, 18 hr. culture	"	0	0	0	0		32 days	0	0
4	Control	None	16,000,000, 18 hr. culture	"	0	0	0	4620		45 hrs.	+	++
5	Actively immune	12 injections, 1 cc. vaccine, intravenously	111,000,000, 18 hr. culture	"	60	810	4020			22 "	+	++++
6	Control	None	111,000,000, 18 hr. culture	"	31,800	∞	∞			22 "	+	+
7	Actively immune	12 injections, 1 cc. vaccine, intravenously	55,000,000, 18 hr. culture	"	0	0	Accidental death			5½ "	+	0
8	Control	None	55,000,000, 18 hr. culture	"	0	920	∞			26 "	+	+
9	Actively immune	12 injections, 1 cc. vaccine, intravenously	81,000,000, 18 hr. culture	"	20	165	375			22 "	+	++
10	Control	None	81,000,000, 18 hr. culture	"	530	∞	∞			22 "	+	++
11	Passively immune	5 cc. N.Y.S. serum intravenously	35,000,000, 18 hr. culture	"	20	535	900			22 "	+	++++

12	Passively immune Control	5 cc. N.Y.S. serum intravenously None	35,000,000, 18 hr. culture	Cistern	0	750	4610	60	44 days	+	++
13			35,000,000, 18 hr. culture	"	55	1600	42,840		22 "	+	+++
14	Passively immune	10 cc. N.Y.S. serum intravenously	26,000,000, 18 hr. culture	"	5	290	2180	60	46 "	+	+++
15	Actively immune	6 injections, 1 cc. vaccine, intravenously	26,000,000, 18 hr. culture	"	0	15	15		26 "	0	+++
16	Control	None	26,000,000, 18 hr. culture	"	2320	∞	∞		22 "	+	++
17	Passively immune	10 cc. N.Y.S. serum intravenously	13,000,000, 18 hr. culture	"	0	0	0		22 "	+	+++
18	Passively immune	10 cc. N.Y.S. serum intravenously	13,000,000, 18 hr. culture	"	30	750	1920		22 "	+	+++
19	Control	None	13,000,000, 18 hr. culture	"	+	10,800	∞		22 "	No autopsy	
20	Passively immune	10 cc. N.Y.S. serum intravenously	9,000,000, 18 hr. culture	"		135	4920	1060	45 "	+	++
21	Passively immune	10 cc. N.Y.S. serum intravenously	4,500,000, 18 hr. culture	"		56	510	10	72 "	0	++
22	Passively immune	10 cc. N.Y.S. serum intravenously	1,000,000, 18 hr. culture	"		80	265	0	72 "	0	+++
23	Control	None	1,000,000, 18 hr. culture	"		1740	12,280	41,600	45 "	+	++
24	Passively immune	10 cc. N.Y.S. serum intravenously	5,000,000, 18 hr. culture	"		0	175	0	70 "	0	+++
25	Passively immune	10 cc. N.Y.S. serum intravenously	5,000,000, 18 hr. culture	"		0	5	0	24 "	0	+++
26	Control	None	5,000,000, 18 hr. culture	"		11,360	16,280		Hemorrhage 46 hrs.	+	+

TABLE I—Concluded.

No.	Type	Method of immunization	Infecting dose	Site of infection	Result of blood cultures					Duration of life	Post-mortem heart blood culture	Meningeal reaction
					1-1½ hrs.	2½-3 hrs.	5-5½ hrs.	24 hrs.	48 hrs.			
27	Passively immune	10 cc. N.Y.S. serum intravenously	6,000,000, 18 hr. culture	Ear vein		0	5	0		Lived		
28	Passively immune	10 cc. N.Y.S. serum intravenously	6,000,000, 18 hr. culture	"		0	0	0		"		
29	Control	None	6,000,000, 18 hr. culture	"		4920	7640	∞		70 hrs.	+	
30	Actively immune	12 injections, 1 cc. vaccine, intravenously	3,000,000, 18 hr. culture	Cistern		0	15	0		Lived		
31	Control	None	3,000,000, 18 hr. culture	"		27,000	∞			27 hrs.	+	+
32	Passively immune	10 cc. N.Y.S. serum intravenously	7,200,000, 18 hr. culture	"	0	60	700			48 "	+	+++
33	Passively immune	10 cc. N.Y.S. serum intravenously	4,800,000, 18 hr. culture	"	5	3640	2680			48 "	+	+++
34	Control	None	2,400,000, 18 hr. culture	"	1400	10,200	18,900			72 "	+	+
35	Normal	"	6,000,000, 18 hr. culture + fibrinogen	"	0	0	0			96 "	+	+
36	"	"	6,000,000, 18 hr. culture + fibrinogen	"	0	0	0			26 "	+	+++
37	"	"	6,000,000, 18 hr. culture	"	2400	∞	∞			22 "	+	+

injections each of heat-killed Type I pneumococci. Each injection consisted of the quantity of organisms contained in 1 cc. of 12 hour broth culture, centrifuged and resuspended to original volume in saline. Agglutination titers in these actively immunized rabbits did not exceed 1:5 after one series of injections, nor 1:20 after two series. Of about 80 animals examined, only one showed agglutinins in the spinal fluid. In a small series of rabbits actively immunized by both intravenous and intracisternal injections of killed Type I pneumococci, no agglutinins were present in the spinal fluids when examined 1 week after the final injection. It would appear, therefore, that any restraint observed in the progress of the pneumococcal meningitis locally in partially immune animals is not due to agglutinating antibodies in amounts capable of titration in the spinal fluid at the time of infection.

A considerable number of animals was immunized in the above manner. Subsequent infection was by direct inoculation of organisms in the cisterna magna. The preliminary experiments on restraining the septicemia were performed with an 18 hour broth culture, producing a growth of about 250,000,000 per cc. In all but the initial experiments the dosage was determined by the use of the Petroff-Hausser counter. Bleedings were made from the ear vein at varying intervals after infection, and the number of colonies per cc. of blood counted. A control non-immune rabbit was used for comparison for all doses of organisms. The results of immunization in controlling the development of the septicemic spread from the meninges are evident in Table I.

In all the experiments summarized in the first table, the organism used was type-specific and capsule-forming, and produced only the smooth variety of colony. In view of the recently developing interest in pneumococcus variants (R forms), it was considered desirable to test out such R forms for meningeal pathogenicity. The R form used was obtained from Dr. Avery and was derived experimentally from the same type-specific strain employed above. It was developed from a single cell colony.

Rough Type I pneumococci were injected intracisternally in eleven rabbits. Some were normal rabbits; others had recovered from *Streptococcus scarlatinæ* meningitis. In all, a transient bacteremia



TABLE II.

No.	Type	Dosage Type I R	Results of blood cultures				Duration of life	Autopsy cultures		Meningeal reaction
			1-1½ hrs.	3-3½ hrs.	5-5½ hrs.	24 hrs.		Heart	Cistern	
38	Normal	100,000,000, 18 hr. cul- ture, cistern	1020	3760	800		24 hrs.	0	+	+
39	"	50,000,000, 18 hr. cul- ture, cistern	300	35	25	0	Lived			
40	"	20,000,000, 18 hr. cul- ture, cistern	5	15	5	0	"			
41	"	100,000,000, 18 hr. cul- ture, cistern	0	160	525		24 hrs.	0	+	+
42	<i>Strep. scarlatinæ</i> ; recovery	100,000,000, 18 hr. cul- ture, cistern	5	5	5	0	Lived			
43	<i>Strep. scarlatinæ</i> ; recovery	100,000,000, 18 hr. cul- ture, cistern	0	30	0	0	48 hrs.	0	+	+
44	<i>Strep. scarlatinæ</i> ; recovery	250,000,000, 18 hr. cul- ture, ear vein	3120	320	300	0	Lived		Very rare	
45	<i>Strep. scarlatinæ</i> ; recovery	100,000,000, 16 hr. cul- ture, cistern	220	3640	35,280	0	14 days (Pneumonia)	0	0	0
46	Normal	100,000,000, 16 hr. cul- ture, cistern	300	13,320	1740		24 hrs.	+	+	+
47	<i>Strep. scarlatinæ</i> ; recovery	100,000,000, 16 hr. cul- ture, cistern	960	440	225	0	7 days (Enteritis)	0	0	±
48	Normal	100,000,000, 16 hr. cul- ture, cistern	240	215	65	0	78 hrs. (Pneumonia)	0	0	+

resulted (Table II). Only once, did the latter persist for 24 hours. Large doses of organisms were used, usually 100,000,000. This dose frequently killed. The mechanism of death is not clear. Few organisms could be detected in cistern fluid smears at death, but they were mostly phagocyted. The edema and congestion of the lungs were quite marked. If death did not occur within 24 hours, recovery was the rule. In a series of eleven animal passages, no reversion to a smooth colony type was observed.

It is obvious from consideration of Table I that active or passive immunization of animals previous to cisternal infection results in a retardation of septicemia. Where, exactly, this retardation occurs is difficult to determine on account of the multiplicity of factors involved, and on account of these variable factors, after a given degree of immunization the extent of the septicemia following intracisternal infection is irregular. The appearance of organisms in large numbers in spinal fluids is delayed. This retardation was especially notable in later experiments on treatment, where frequently, after an injection of upwards of 3,000,000 organisms intracisternally, the 19 hour cisternal fluid showed rare pneumococci only, and where in one instance spontaneous recovery of an untreated immune rabbit occurred, when all treated animals of that group died. It is likewise evident in early experiments (Table I), that in actively immune animals much larger infecting doses are required to produce a fatal meningitis. So far as was determined, this local meningeal retardation does not depend upon the presence of agglutinating antibodies in the fluid at the time of infection, although their ultimate influence is not beyond the realm of possibility, since following the cisternal injection, choroid plexus alteration may result in some diffusion of agglutinating antibodies present in the blood, or of opsonins (7). We have not done any opsonic studies on spinal fluids of immune animals.

In partially immune rabbits pneumococcal meningitis is occasionally abortive, but it would appear that in the meninges, doses of organisms will produce progressive disease and also eventual septicemia, whereas several times the number of diplococci injected intravenously will produce nothing at all. Therefore it may be assumed that the meningeal spaces constitute to some, and probably to a considerable extent, a non-immune locus, where organisms in numbers insufficient to

produce systemic disease may multiply, pass the filtration mechanism in great numbers, and break down the systemic resistance.

After the series of preliminary experiments outlined in Table I, we determined to try the effect of antisera in the control of the meningeal disease. Not much hope was entertained, in view of the severity of the process, but it was thought that if any evidence at all indicated benefit from serum therapy in so extremely susceptible an animal as the rabbit, there would be some justification in advocating its utilization. In the majority of instances, the concentrated antibody solution prepared for us by Dr. Lloyd Felton, of the Harvard Medical School, was used. According to Dr. Felton's titration, the samples employed by us contained 50 and 3000 protective units per cc. We must acknowledge that so far as beneficial therapeutic results are concerned, we were unable to see any difference in the two strengths of antibody solutions, nor could we detect that either differed appreciably from the Type I antipneumococcus serum prepared in the New York State laboratories. It is impossible to express results of therapeutic experiments in tabular form, and since it is not desirable to include all protocols, a short series only of the latter is included. It may be emphasized here that, whereas for convenience in planting and timing cultures, an 18 hour growth was used in preliminary studies of the septicemia, in therapeutic experiments a 6 hour culture was used. The first culture had obviously gone beyond its developmental peak, while the second was still in an active growth phase. It became apparent early that the second culture was much more active and that with given immunization a smaller dose of the 6 hour culture would duplicate the results of a larger 18 hour culture. It was likewise noted that the apparent vigor of the culture, as expressed by actual growth per cc. in a given number of hours, was also very important. Since the actual dosage of organisms in each instance was counted, we are forced to conclude that there must have been either an actual variability of potency in the individual cocci under unfavorable growth conditions, or possibly that the total constituency of the culture varied, cultures giving poor growth containing more damaged or dead cocci. These factors may be of minor importance in influencing ultimate fatal disease in normal rabbits, but may play a considerable rôle where partial immunity has been established. Then again, there

is an uncontrollable individual variation in animals; given a certain dosage of immune serum and a certain known cisternal dosage of pneumococci from the same culture, results will vary. These varying factors are shown in the following few representative protocols.

### *Illustrative Protocols.*

*Rabbit A.*—Active immunity established by intravenous and intracisternal vaccine. Immunization completed December 4, 1926. December 14, 1926, 4.00 p.m., ether, cistern puncture, clear fluid; injected  $17\frac{1}{2}$  million Type I pneumococci. Good recovery. December 15, 9.30 a.m., temperature below 92°. Cistern puncture, rare cells but a massive, diffuse overgrowth of pneumococci. No phagocytosis. Injected 1 cc. Felton 50 unit antibody solution intracisternally, and 5 cc. antipneumococcus serum intravenously. Placed on electric pad. Temperature recovery to 100.2°. Dead 2.30 p.m. Autopsy: Brain is pale, meninges slightly opalescent. Cisternal fluid, instead of rare cells observed previous to treatment, contains, on smearing, from 60–100 polymorphonuclears per oil immersion field. Very active phagocytosis of pneumococci; free organisms largely agglutinated. Culture: massive growth; heart blood: no growth. Microscopically: fibrinopurulent exudate over cerebrum, cerebellum, medulla, and cord; exudate also involves dura, choroid plexuses, cord septa, sheaths of penetrating vessels. Large number of pneumococci. Phagocytosis confined essentially to region of cisterna. Moderate superficial encephalitis. Note: an example of massive proliferation of pneumococci in the meninges, no terminal septicemia, an initial poor cellular reaction in meninges, greatly improved by antibody injection, and induction of marked phagocytosis.

*Rabbit B.*—Same immunization and infecting dosage as in Rabbit A. Infected same day with same culture. 18 hours after infection, temperature 103.2°, sluggishness, fine tremors; 22 hours, hind limbs paralyzed. Cistern puncture under ether; cells 4–12 per o. i. f., moderate number of cocci, no phagocytosis. Injected 1 cc. 50 unit antibody, cisternally, and 5 cc. antiserum, intravenously. Temperature fell to 95°. 24 hours, second cistern puncture; cells 20–100 per o. i. f.; rare agglutinated cocci. First culture, massive growth; second, few colonies only. 42 hours: cisternal fluid contained large numbers of cocci. Injected 2 cc. antibody, with symptoms of pressure. 66 hours: fluid as above. Injected 1.5 cc. antibody. Death in 2 hours. Autopsy: Similar to Rabbit A, save that the lumbar cord is essentially gangrenous. Cisternal cell number again markedly increased (60–100 per field); agglutination and phagocytosis of cocci. In addition to usual microscopic findings, considerable periarteritis of larger vessels of cord meninges, purulent ventriculitis of third and lateral ventricles, abscesses of choroid plexuses of fourth and lateral ventricles, and ingrowth of cocci into wall of lateral ventricle. Summary: Same immunization and dosage, but a considerably lesser initial process. Repeated treatments always

resulting in increased cellular reaction and phagocytosis, but in the interim between treatments great augmentation of cocci from regions untouched by treatment. Heart blood: no growth.

*Rabbit C.*—Immunization as in the two preceding. December 16, 1926, 4.00 p.m., ether, cistern puncture, clear fluid. Injected 12 million Type I pneumococci, 7 hour culture. 18 hours later, temperature 106.5°. Cisternal fluid opalescent, cells 10–20 per o. i. f.; only occasional clusters of diplococci; culture slight growth. By vital staining cells 64 per cent polymorphonuclears, 34 per cent monocytes, 2 per cent small lymphocytes. Injected 1.5 cc. antibody solution. 23 hours, lumbar and cistern punctures; leucocytes increased; very few phagocytic cocci; none free. Cultures: no growth. Injected 1 cc. antibody in cistern and in lumbar cord. No further treatment and an uneventful recovery. Summary: Delayed development of meningeal infection with few organisms in fluid at time of treatment. Rapid sterilization of a mild process. Recovery not necessarily due to treatment since immunization may have played the more decisive rôle.

Second animal of this group, similarly treated, developed a massive growth of cocci and died in 41 hours with only moderate meningeal reaction and a slight septicemia. These two rabbits emphasize the individual factor. A control, actively immune, untreated rabbit died in 41 hours with severe septicemia and a good meningeal reaction.

*Rabbit D.*—December 13, 1926, 9.50 a.m., 10 cc. Type I antipneumococcus serum, ear vein. 4.10 p.m., ether, cistern puncture, clear fluid, injected 3,000,000 Type I pneumococci, 7 hour culture. 18 hours after infection temperature 106.4°. Cisternal puncture; fluid slightly opalescent; cells very rare and only occasional cocci. Culture slight growth. Injected 1 cc. 50 unit antibody. 24 hours after infection second cistern puncture; fluid abundant, almost purulent; polymorphonuclears about 100 per o. i. f.; no cocci seen; culture negative; 1 cc. antibody injected. 42 hours, temperature 105.4°; active. 66 hours: appears slightly ataxic and has a head nystagmus. Spinal fluid culture negative. 5 days: temperature 104.5°; improved. 7 days: unchanged. 10 days: found dead. Autopsy: Spinal cord and brain pale, meninges opalescent. Cisternal fluid seropurulent, containing 50–100 polymorphonuclears per o. i. f. and a diffuse overgrowth of pneumococci. Marked congestion and edema of lungs. Culture of heart blood, no growth. Usual histology of fibrinopurulent meningitis. Summary: Low grade initial process in passively immune rabbit; culture of cisternal fluid twice negative after antibody injections. Final recrudescence of meningeal infection leading to delayed death at 10 days.

A second rabbit of this group, similarly immunized and infected, developed a massive growth of pneumococci within the first 18 hours, with essentially no cellular reaction. The latter was greatly improved by 1 cc. of antibody and at death, 3 hours later, there were large numbers of leucocytes and phagocytosis of 50 per cent of the organisms in the cisternal fluid. Blood cultures gave moderate growth.

The passively immune, untreated control died of meningitis and septicemia in 36 hours.

*Rabbit E.*—December 20, 1926, 10.00 a.m., 10 cc. Type I antipneumococcus serum ear vein; 4.00 p.m., 3,000,000 Type I pneumococci intracisternally. 18 hours: temperature 106.2°; active. 42 hours: temperature 106.3°; sluggish; diagnostic cistern puncture; cells rare; pneumococci very rare; culture positive; blood sterile. 66 hours: temperature 105.4°; sluggish; marked ataxia and weakness; diagnostic cistern puncture; essentially no change in fluid picture. 88 hours: dead. Petechial hemorrhages over cerebellar hemispheres; meninges opalescent, brownish, gelatinous looking exudate at base; cord not grossly remarkable; cisternal fluid smear shows about 100 leucocytes per o. i. f. and a large diffuse overgrowth of diplococci. Practically no phagocytosis. Microscopically: a thick fibrinopurulent meningitis. Summary: A markedly delayed initial process with essentially no early cellular reaction; a terminal extensive leucocytosis with little phagocytosis and a diffuse coccal overgrowth.

Of this group two animals were treated; both had mild initial processes at 18 hours and were lavaged from lumbar to cistern with antibody solution. One died at 88 hours with a pneumonia from which Type I pneumococci were isolated; at death both spinal fluid and heart blood gave no growth. The other animal recovered. Although this effect would appear to indicate favorable results of treatment, in our opinion it means nothing. There are too many variable factors involved to make correct analysis possible.

In recovered rabbits, various postmeningitic signs may occur. Prominent among these are paraplegias, ataxia, nystagmus, and prolonged opisthotonus. With the exception of the paraplegias, there is a strong tendency to amelioration of these conditions.

Whereas in small series of three to five rabbits, similarly immunized and infected, controls may die and treated animals recover, in one instance the reverse has been true, four treated animals dying, and one untreated recovering. This occurrence only goes to prove the impossibility of evaluating results when such variable factors are present. Individuals react differently after the same immunization; there is a very narrow zone between a dose of organisms which in a partially immune animal may produce a massive growth of cocci in the meninges with or without septicemia, and a dose which may permit spontaneous recovery. If we take length of life as an index of value of therapy, it is apparent that the treated animals dying, lived an average of 24 hours longer than the controls; if we consider the presence or absence of septicemia, it becomes evident that 28 per cent of animals treated locally and systemically subsequent to infection had positive blood cultures at autopsy and that 70 per cent of partial immunes with no subsequent treatment had terminal septicemia. Length of life in treated animals is difficult to interpret on account of a high percentage of traumata with repeated punctures; working space is very small in the rabbit, and the needle permitting satisfactory lavage is of course disproportionately large. Reac-

tion to intracisternal injection of antibody solution is often pronounced. We may summarize it in the following protocol.

*Rabbit F.*—Actively immune; uninfected; temperature 102.5°. Ether; cistern and lumbar puncture; clear fluid. Lavage with 2 cc. antibody solution, strength 50 units per cc. Immediate ether recovery. Within 10 minutes, prostration, tremors, chill, progression movements of all four limbs, rapid, shallow respiration, temperature 95.7°, falling in 2 hours to 92°. Following morning slightly sluggish, temperature 99.5°. Subsequent uneventful recovery.

In one instance the reaction did not begin until 1½ hours after injection of antibody. Samples of antibody containing no tricesol produced at times similar effects. These reactions may be duplicated by trauma to medulla, but frequently no trauma could be verified at autopsy. In the uninfected animal intrathecal antibody solution causes a slight transient polymorphonuclear reaction, rapidly passing over to an exudate containing small lymphocytes and endothelial leucocytes.

### *Pathology.*

The microscopic appearances of the pneumococcal meningitis have been studied in all the animals. The pathology is distinctly a function of immunization, dosage, and treatment. At one extreme one has the non-immune rabbit infected *via* the cisterna. The tendency in this animal is for the cerebrospinal fluid to be turned into a veritable culture medium for pneumococcus (Fig. 3); the pia-arachnoid is filled with a massive sheet of pneumococci; the choroid plexuses contain them in great numbers; they invade the Virchow-Robin spaces in solid masses (Fig. 4); they multiply within the ventricles. In this form of disease there is very little reactional change in the meninges; death is always with marked septicemia usually complicated by edema and congestion of the lungs. In a partially immunized rabbit death is delayed, and time is allowed for a vigorous cellular reaction in the meninges; this takes the form of a thick, fibrinopurulent exudate, involving the meninges of all regions, cord, cerebellum, convexity, base and choroid plexuses. Its intensity is variable. Pneumococcal proliferation may be restrained at first, but eventually usually reaches an extreme degree, the cocci lying in solid masses between reacting cells (Fig. 5); there is little phagocytosis. If there is intrathecal treatment, an increased leucocytosis, with active phagocytosis, agglutination, and thread reaction result (Figs. 6-8). Phagocytosed cocci stain supravitaly with neutral red. These phenomena are but transitory and

within a few hours there is a tendency for cells to diminish and for the diffuse growth of organisms to be resumed. This constant renewal of pneumococci originates from agglutinated, unphagocytized clusters of pneumococci and particularly from regions untouched by treatment. It is evident that it is impossible to reach, by treatment, all regions in the rabbit, if the initial growth of bacteria is at all severe. Exudate with pneumococci may be found in the central canal of the cord, between fibers of issuing nerve trunks (Figs. 9 and 10), or in spinal ganglia (Fig. 11). There may be empyema of the central canal of the cord, with destruction of the ependyma and involvement of either the cornua or funiculi (Fig. 12). Pneumococci in the sheaths of penetrating vessels are commonly observed. In any severe process, one is apt to find a superficial encephalitis (Figs. 13 and 14), or else foci of softening and invasion by pneumococci (Fig. 15). Periarteritis is common (Fig. 16). Purpuric hemorrhages may occur either in the meninges or deep in the brain or cord substance (Fig. 17). Death is almost always complicated by edema and congestion of the lungs. There may be a considerable fibrin deposit and if life is sufficiently prolonged, a lobular consolidation may result (Figs. 18-20). Pneumococci may be demonstrated in this exudate by Gram-Weigert staining.

#### SUMMARY AND CONCLUSIONS.

1. Fatal Type I pneumococcal meningitis may be produced in rabbits by intracisternal injection of pneumococci.
2. When organisms are of high virulence, the rabbit does not tend to localize them in the meninges, but an early septicemic process results. Death is septicemic rather than meningeal.
3. In such instances very little cellular reaction occurs in the meninges.
4. Active or passive immunization previous to intracisternal infection inhibits partially the septicemia and permits the development of reactional processes in the meninges.
5. The immunization likewise retards the meningeal disease, but multiplicity of factors prevents us from stating precisely to what this retardation is due. It is not correlated with the presence of agglutinins in the spinal fluid at the time of infection.
6. The rapidity of production of meningitis is influenced by the



phase of growth of the culture used, and likewise by the growth activity of that culture.

7. To some extent in the partially immune rabbit the meningeal spaces constitute a relatively non-immune reservoir, constantly feeding the blood stream and breaking down systemic resistance.

8. Intrathecal serum treatment causes rapid agglutination and phagocytosis of pneumococci, and has very rarely, possibly, resulted in cure. Essentially no phagocytosis occurs in the absence of immune serum.

9. Phagocytosed pneumococci stained supravivally take up the neutral red stain and are therefore probably injured.

10. The treatment employed subsequent to infection only slightly prolongs life in the majority of cases. It does retard septicemia.

11. The treatment improves the cellular reactional processes in the meninges.

12. A study of the pathology of rabbit pneumococcal meningitis shows that the location of pneumococci precludes complete contact with serum introduced intrathecally, and that these locations provide isolated foci, from which organisms may reinfect the meningeal spaces as rapidly as they are removed by lavage or antibody injections.

13. In recovered rabbits postmeningeal symptoms, weakness, ataxia, nystagmus, and paralyzes arise.

14. In our opinion there is some objective evidence of benefit of serum therapy. The rabbit is too susceptible, however, and conditions too artificial to admit of definite conclusions.

15. Rough Type I pneumococci introduced in large quantity cisternally may kill and may be recovered 24 hours after infection, from spinal fluids. In a series of eleven passages, no reversion to smooth type occurred. In all animals injected with rough forms, transient bacteremia resulted.

#### BIBLIOGRAPHY.

1. Lamar, R. V., *J. Exp. Med.*, 1912, xvi, 581.
2. Lamar, R. V., *J. Exp. Med.*, 1911, xiii, 1.
3. Kolmer, J. A., *Arch. Otolaryngol.*, 1926, iii, 481.
4. Kolmer, J. A., and Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 355.
5. Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 373.
6. Untersteiner, R., *Z. ges. Neurol. u. Psychiat.*, 1926, cii, 64.
7. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1927, xlv, 239.

## EXPLANATION OF PLATES.

## PLATE 11.

FIG. 1. Non-immune rabbit. Pneumococci in lung capillaries. Pulmonary edema. Gram-Weigert stain.  $\times 1000$ .

FIG. 2. Same animal, stain, and magnification. Pneumococci in capillary of renal medulla.

FIG. 3. Spinal fluid smear. Shows type of fluid found in a non-immune rabbit or in one partially immune, which has developed a massive overgrowth of cocci previous to treatment.  $\times 1000$ .

FIG. 4. Non-immune rabbit. Solid masses of pneumococci in perivascular sheath of penetrating cortical vessel. Gram-Weigert stain.  $\times 1000$ .

## PLATE 12.

FIG. 5. Massive growth of pneumococci in partially immune treated rabbit. Slight phagocytosis. Section of cord far distant from region of treatment. Gram-Weigert stain.  $\times 1000$ .

FIG. 6. Agglutination and vigorous phagocytosis in exudate of treated animal.  $\times 1000$ .

FIG. 7. Thread reaction in agglutinated cocci after treatment.  $\times 1000$ .

FIG. 8. Phagocytosis of pneumococci in exudate of treated animal. The same animal as in Fig. 5, but from region near location of treatment. Gram-Weigert stain.  $\times 1000$ .

## PLATE 13.

FIG. 9. Polymorphonuclear invasion of spinal nerve trunk.  $\times 260$ .

FIG. 10. Growth of pneumococci between fibers of spinal nerve trunk. Non-immune rabbit. Gram-Weigert stain.  $\times 1000$ .

FIG. 11. The same animal, stain, and magnification. Pneumococci in a spinal ganglion.

FIG. 12. Cord abscess (pneumococcal) in a partially immune rabbit; an extension from empyema of central canal of cord.  $\times 150$ .

## PLATE 14.

FIGS. 13 and 14. Superficial encephalitis. Partially immune rabbit.  $\times 260$ .

FIG. 15. Pneumococcal invasion with softening of wall of lateral ventricle. Treated animal dying at 21 hours. Gram-Weigert stain.  $\times 1000$ .

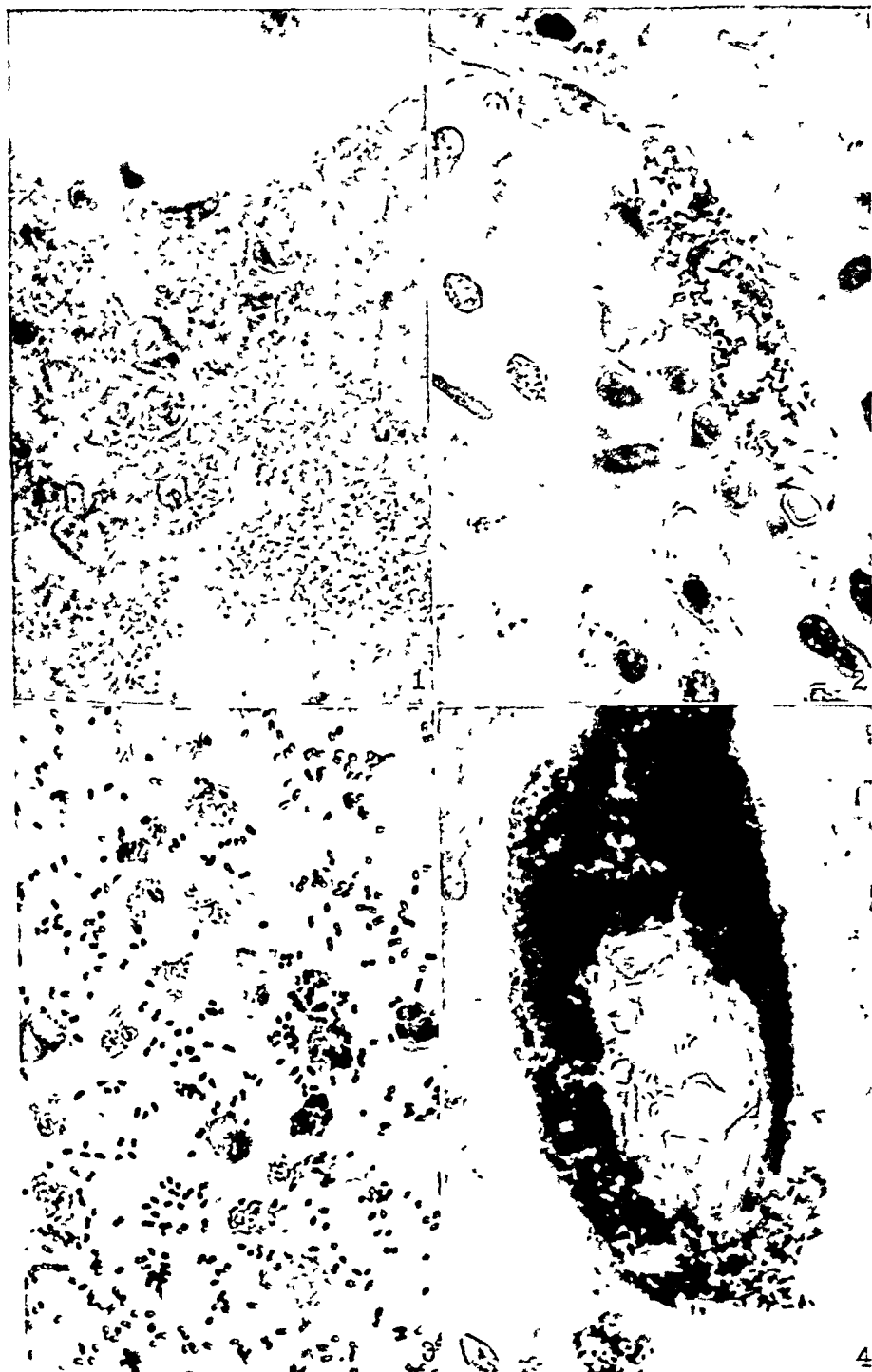
FIG. 16. Periarteritis, meningeal cord vessel. Partially immune rabbit.  $\times 260$ .

## PLATE 15.

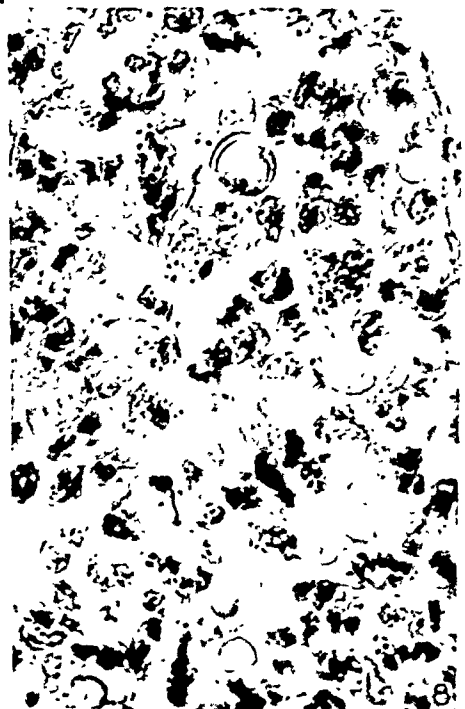
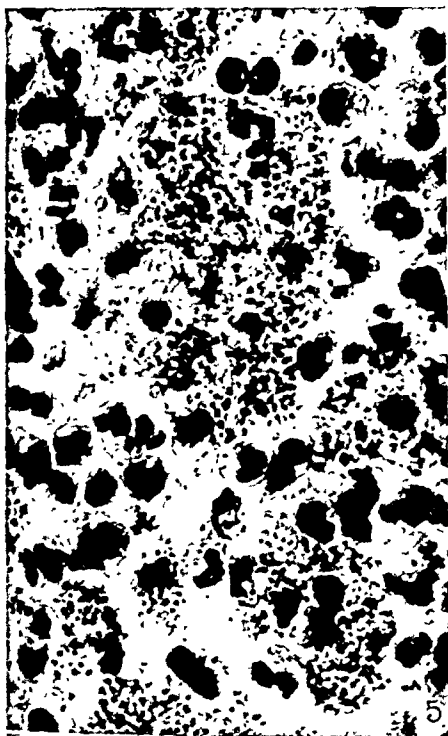
FIG. 17. Hemorrhagic lesion; gray matter of cord. Partially immune rabbit dead 28 hours after infection.  $\times 110$ .

FIGS. 18 to 20. Pneumococcal pulmonary lesions as observed in partially immune rabbits.  $\times 50$ .

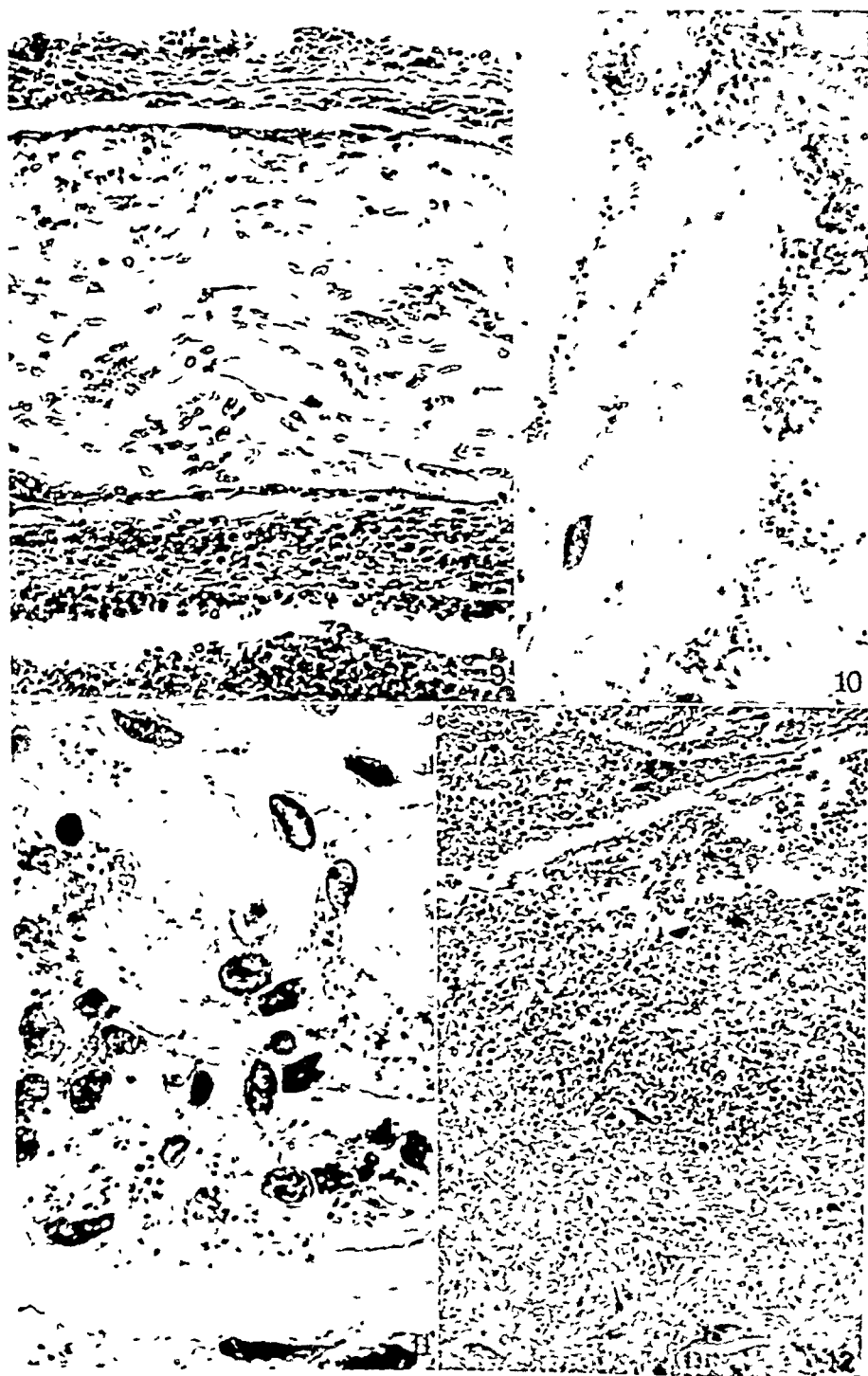






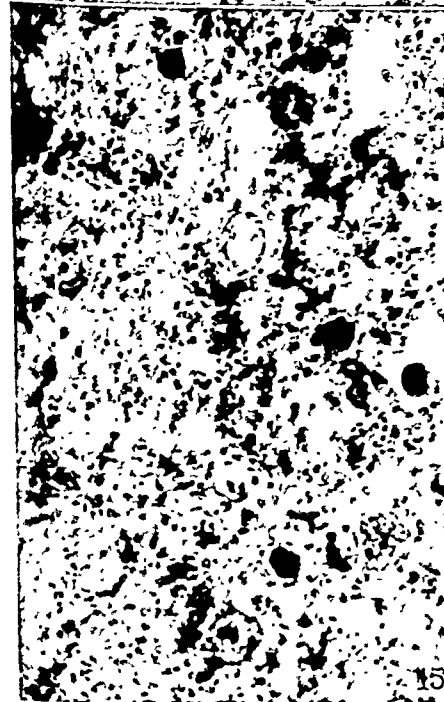




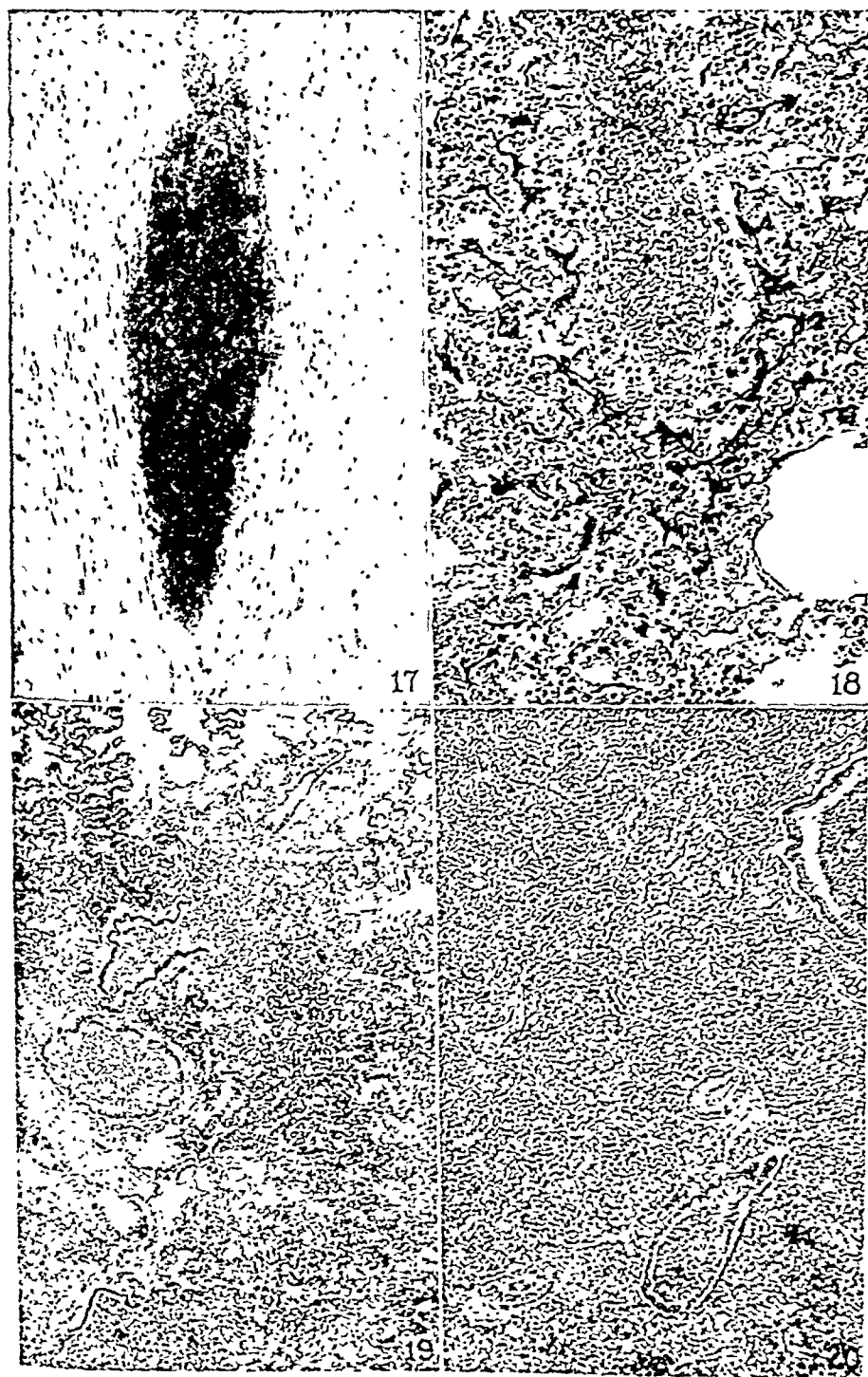














# LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

## II. THE PRODUCTION, PATHOLOGY, AND TREATMENT OF TYPE I PNEUMOCOCCAL MENINGITIS IN DOGS.

By FRED W. STEWART, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 16 TO 21.

(Received for publication, June 12, 1927.)

In the previous paper (1) the production, pathology, and attempted treatment of Type I pneumococcal meningitis of rabbits were discussed. Reasons were presented explaining why the rabbit is a very unfavorable animal for the study of this disease, which may be briefly summarized as follows: In a normal rabbit, septicemia, following intracisternal infection with Type I pneumococci, is invariable, rapid, and massive, and septicemic death occurs before there is any reactional localization in the meninges; consequently, to produce meningeal localization, complete or partial, a previous immunizing treatment is necessary. Following this treatment, however, there is a considerable variation in the intensity of the meningeal infection, making it impossible to gauge properly the dosage of organisms which will produce fatal disease, but which will not immediately overwhelm the animal's resistance. Even if the disease intensity is properly regulated, treatment is unsuccessful on account of both the limited working space and the widespread localization of organisms in regions impossible to reach either by immune serum or lavage.

In view of these facts, the rabbit was abandoned as an experimental animal and recourse was had to young dogs. The dog is, as we know, relatively resistant to systemic pneumococcal infection, although marked variations even here are encountered. In the meninges, however, a type of disease may be produced which progresses toward fatal termination, if cultures of suitable virulence are used, and one which quite closely approximates pathologically the human pneumococcal

meningitis. To produce rapid progressive meningitis with any degree of assurance that the disease will assume a fairly characteristic course from day to day, the infecting dose must be reasonably standardized. In the present study the dosage of organisms has been accurately counted by means of a Petroff-Hausser bacterial counter. Not only should the numbers of infecting pneumococci be known, but the culture should be standardized both as to hours of growth and as to quantity of growth. A culture should be chosen which is well along on the upward limb of the growth curve. We have employed 6 hour cultures. It must be a culture which has given a good growth. There is a marked difference between the degree of 18-24 hour infection produced by a given number of pneumococci from a 6 hour plain broth culture which has produced a low total growth, *i. e.*, a growth ranging from 150-225 million per cc., and that produced by the same number of organisms from a 6 hour 0.2 per cent dextrose broth culture which is producing a growth of upwards of 700 million per cc. Unless these differences are recognized, conclusions are of little value. Use of late (24 hour) cultures may lead to very deceptive results. Even with these cultural conditions standardized, there is an uncontrollable individual factor which influences the rate of establishment of progressive disease; every animal is an individual and must be so handled. Whereas we hesitate to interpret the anatomic and therapeutic results of others, we are inclined to believe that the findings of Idzumi (2) were the result of the use of a culture of low virulence. Idzumi studied pneumococcal meningitis in dogs. This investigator used enormous doses of organisms, from 1-2 cc. of a suspension secured by centrifuging 10 cc. of a 24 hour culture of low mouse virulence. Although the dogs died, the pathologic changes were scarcely more than those of hyperemia. In our own study the disease has been regularly produced with approximately 1 cc. of a 1:100 dilution of 6 hour culture. It would seem that Kolmer's (3) successful therapy, *i. e.*, lavage from one or both lateral ventricles to the cisterna magna with physiological saline or Ringer's solution, cannot be interpreted as the result of other than feeble infection with cultures well beyond the growth peak and full of non-viable organisms. The lavage was most incomplete, since the entire cord, base, and convexity were untouched by the saline. Kolmer reports, however, that at the time of lavage

the spinal fluids were always purulent and contained myriads of pneumococci, making it more difficult for one to understand how lavage from *one* lateral ventricle to the cisterna magna could have appreciably influenced the process, since in our own experience the other ventricle would always have been an untouched focus in a disease of the intensity he describes. Bull (4) observed meningitis developing in the course of pneumococcal septicemia in dogs. His organism was not typed. Bull noted some spontaneous recoveries.

#### EXPERIMENTAL.

Apparently in control dogs, infected intracisternally, death may occur in one or more of three ways. A dog may develop a fairly rapid proliferation of pneumococci in the meninges with a constant large feeding of organisms into the blood stream, and die of bacteremia. If a culture is of low potency due to poor growth conditions, a meningeal process of such intensity may result that a very marked cellular reactional process occurs, with pneumococci present in relatively small numbers. In such cases death appears to be "reactional." Thirdly, the proliferation of organisms within the meninges may attain enormous proportions, and brain and cord may be covered with a thick, gelatinous layer, consisting mainly of heavily encapsulated pneumococci, but with relatively moderate cellular reaction. In the second and third instances, death may be due, in part at least, to the mechanical obstruction of the meningeal spaces. The actual mechanism of death from pneumococcus infection is always rather obscure. Three protocols may be introduced to illustrate these points.

*Dog 12.*—Female hound; weight 5.5 kilos. March 23, 1927, 4.10 p.m., morphine gr. 1/5; ether; cistern puncture; clear fluid. Injected 6,500,000 Type I pneumococci (6 hour dextrose broth culture, growth 1,100,000,000 per cc.). Good recovery. 19 hours later, lethargic, weak, irritable, sicker than usual at this stage. 23 hours, morphine gr. 1/5; death in 10 minutes. Autopsy: Brain and cord injected, edematous; cisternal fluid cloudy; marked grayish yellow opacity along sulci of convexity and at base. Smears from the usual regions show on an average 20–30 pneumococci per oil immersion field and 30–60 cells, mostly polymorphonuclears. Viscera negative. Culture from heart's blood gave massive confluent growth. A death mainly due to septicemia. Microscopically, however, there was a marked diffuse fibrinopurulent leptomeningitis.

*Dog 3.*—Female hound; weight 7.5 kilos; February 9, 1927, 4.20 p.m., ether,



cistern puncture, clear fluid. Injected 15,000,000 Type I pneumococci (7 hour plain broth culture giving poor growth). Good recovery.

23 hours—temperature 103°. Sluggish; refuses food.

43 hours—temperature 100.2°. Unchanged; 100 cc. saline intraperitoneally.

67 hours—temperature 103°. Sleeps most of time; able to stand; 75 cc. saline intraperitoneally.

93 hours—temperature 102.2°. Able to stand leaning against cage; weak; stiffness of neck; 75 cc. saline.

5th day—temperature 102°. Very irritable.

6th day—temperature 103.2°. Irritable; chills; tremors.

7th day—temperature 99.8°. Prostrate; hypersensitive; tremors; incontinence of urine and feces; 100 cc. intraperitoneal saline.

8th day—dead. Autopsy: Upon opening spinal cord and cranial cavity, meninges are opaque and pinkish yellow; purpuric blotches in region of medulla. Cord dura is very tense; on slitting it, thick, grayish or greenish yellow pus exudes; this is generalized in distribution, but most abundant in lower medullary, upper cervical, and region of lumbar enlargement. Smears show thick masses of polymorphonuclear leucocytes and rare endothelials. Only occasional diplococci, all extracellular. Over the cerebellar vermis and adjacent portions of cerebellar hemispheres is thick pus. A smear shows a similar picture to that from cord. The base of the medulla and the pons is coated with thick, greenish yellow pus (Fig. 1). Much less exudate over convexity, and pneumococci are rare. The lateral ventricles are dilated and filled with thick pus, and the walls appear eroded. In the ventricular pus cocci are more numerous, but still do not reach the usual proportions; are free and in small agglutinated clusters. Essentially no phagocytosis. Heart's blood cultures are negative.

Summary: Infection with culture giving poor growth; delayed death; enormous reactive process; few pneumococci at autopsy. Suggests a "reactional" death. Histologically a very marked meningitis, superficial encephalitis and myelitis.

*Dog 7.*—Male fox terrier; weight 6 kilos. March 9, 1927, 4.05 p.m., ether; cistern puncture; clear fluid. Injected 7,000,000 Type I pneumococci (6 hour dextrose broth culture, growth 700 million per cc.). Good recovery.

18 hours—temperature 103.5°. Stands, wags tail, refuses food and water.

42 hours—temperature 103.4°. Semiprostrate; 100 cc. intraperitoneal saline.

66 hours—very irritable; unable to stand.

90 hours—dead. Autopsy: Fourth ventricle distended with thick, gelatinous material; cord meninges and meninges over convexity and base tense, opaque, filled with similar yellowish gelatinous deposit. Lateral ventricles contain thick pus. Smears from all regions show numerous pus cells, but an incredible number of pneumococci (Fig. 2), the latter really making up most of the gelatinous material. Essentially no phagocytosis. Both cerebrospinal fluid and heart's blood give abundant growth.

At this point, the observation should be emphasized that smears taken with a loop from different regions of brain or cord meninges give rather deceptive results so far as numbers of exudative cells are concerned. Probably this is due to the fact that cells lie in fibrin meshes wherein they are held. Pneumococci may be picked up more easily and smears, so far as numbers of bacteria go, check fairly well with microscopic findings in sections.

### *Therapeutic Experiments.*

After a few trials of the culture to determine a proper infecting dose, attempts were instituted to treat the animals. The first series consisted of three dogs, two treated and one control. The culture used was that described in the protocol of Dog 3 (above). This culture was of low virulence, producing a prolonged process in the control animal. The infecting dose consisted in 2,000,000 organisms per kilo of body weight; animals were injected intracisternally. It so happened that both treated dogs made uneventful recoveries, save that one had a residual deafness. These animals were treated at an early period in the work, really before we had had much experience in puncturing the cistern and lumbar subarachnoid space, and the daily progress of the disease was not sufficiently followed, nor was the condition of the fluid of one of the treated animals determined previous to treatment. The fluid of the other animal showed but 4-6 cells per oil immersion field, and but 4 diplococci in the entire smear; the culture was nevertheless positive. Treatments were in one instance with Felton's 3000 unit antibody solution, and in the other with unconcentrated antipneumococcus serum. The details of these treatments are not included, since from the behavior of the culture and of the control animal, we feel inclined to disregard this experiment. Suffice it to say that no subsequent test with really virulent culture has promised any duplication of this favorable result. The following protocol summarizes a more systematic study of the effect of treatment, and is introduced to show the behavior of a treated dog for which Dog 7, the animal described above in illustration of the massive pneumococcal overgrowth type of disease, was the control.

*Dog 6.*—Female bull terrier; weight 5 kilos. March 9, 1927, 3.50 p.m., ether; cistern puncture; clear fluid. Injected 7,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 700 million per cc.). Good recovery.

18 hours—temperature 104.7°. Stands, wags tail, refuses food and water.

19 hours—ether; cistern puncture; lumbar puncture; lavage from lumbar to cistern with 20 cc. warm saline, followed by 15 cc. antipneumococcus serum; 5 cc. 800 unit antibody solution intravenously. Cisternal fluid opalescent; looks like a suspension of cocci. Smears of this fluid as follows:

Before lavage—cells 20–30 per oil immersion field; myriads of cocci.

After 15 cc. NaCl lavage—cells rare; cocci about 80 per o. i. f.

After 20 cc. NaCl lavage—cells rare; cocci about 60 per o. i. f.

24 hours—temperature 103°; ether; cistern puncture; seropurulent fluid; drainage. Injected 5 cc. antipneumococcus serum. Smear: numerous pus cells; cocci largely agglutinated and phagocyted; still numerous, but less than at the end of the previous lavage. 75 cc. intraperitoneal saline. Culture: confluent growth.

42 hours—temperature 101.6°. Weak, sluggish, able to stand. Ether, combined puncture; cloudy yellowish fluid; 20 cc. saline lavage, followed by 10 cc. antipneumococcus serum. Smear: before lavage, polymorphonuclears 30–150 per o. i. f.; cocci not increased over previous smear. After completing lavage, both cells and organisms very rare. Culture: confluent growth.

66 hours—temperature 101.7°. Refuses to stand; lethargic; shortly after coming to laboratory a generalized convulsion with salivation and gnashing. Series of bloody stools. Ether; combined puncture; cells 3–60 per oil immersion field; cocci diminished; many look swollen; bacillary; coccoid. 10 cc. antipneumococcus serum.

90 hours—dead. Autopsy: Cord grossly normal; vessels in region of cerebellum and medulla injected; posterior convexity injected; fibrinopurulent material over anterior convexity and base. Thin seropurulent material in lateral ventricles. Smears: cistern—cells 125 per o. i. f.; cocci largely phagocyted. Cord—cells 50 per o. i. f.; cocci very scanty. Anterior convexity—cells 200 per o. i. f.; cocci diffuse with only slight phagocytosis. Lateral ventricles—cells 30–40 per o. i. f.; cocci diffuse with marked phagocytosis. Heart's blood culture—no growth. Viscera: acute intussusception (the cause of death). Microscopically the cord showed relatively little exudate, but the latter was very abundant over the convexity and cerebellum and in the fourth ventricle.

Summary: A very marked initial growth of cocci; clearing by repeated lavage; abundant phagocytosis of residual cocci following antiserum injections but a focus, especially over the anterior convexity, not appreciably reached by serum and thereby constituting a site for subsequent "reinfection" of other regions. Objectively a very decided benefit from treatment.

In view of the fact that it appeared quite possible to lavage certain regions of the central nervous system relatively free from organisms

and to reduce residual pneumococci by the phagocytosis resulting from the injection of immune serum, it was considered desirable to extend, if possible, the field of action of the serum to those portions of the brain not reached by lumbar or cisternal injections. It was determined to treat the convexity by subdural serum injections, after trephining over the frontal lobes. The question arose as to whether one trephine was sufficient, or whether the falx constituted such a complete barrier that bilateral trephining was necessary. To settle this point a single frontal trephine was made just above the frontal sinus in a normal dog under ether anesthesia and 2 cc. of methylene blue was injected. The animal later received likewise 2 cc. intracisternally. The methylene blue injected frontally passed rapidly to the cisterna and with the additional cisternal injection was almost immediately recovered from the lumbar subarachnoid space. The dog was etherized after 1 hour. Staining of the brain was, as far as the convexity was concerned, sharply limited to the hemisphere of the injected side (Fig. 3). This made it obvious that to treat the infected convexity bilateral trephine openings were necessary. The following protocol summarizes one of the early animals treated by quadruple puncture.

*Dog 10.*—Fox terrier, female; weight 7.5 kilos. March 18, 1927, 3.15 p.m., ether; cistern puncture; clear fluid. Injected 8,400,000 Type I pneumococci (6 hour dextrose broth culture; growth 1,400,000,000 per cc.). Good recovery.

21 hours—temperature 99.8°. Sluggish; refuses to stand; will not drink. Ether; lumbar puncture; cells 3–5 per o. i. f.; no cocci seen, but culture positive. Cistern puncture; cells 15–25 per o. i. f.; rare cocci; culture, marked growth but not confluent. Lavage lumbar to cistern with 15 cc. saline followed by 20 cc. anti-pneumococcus serum. Cistern needle withdrawn and left frontal trephine done. Injected very slowly 6 cc. serum. In view of the few organisms in the smear, it was felt that a second frontal trephine was not necessary.

45 hours—temperature 103.6°; unchanged clinically. No treatment.

66 hours—temperature 103.7°; clinically unchanged.

70 hours—diagnostic lumbar puncture; cells 3–5 per o. i. f.; cocci numerous.

72 hours—cistern and lumbar punctures; lavage of 15 cc. saline lumbar to cistern; followed by 15 cc. serum. First cisternal smear shows several hundred pneumococci per field; smear following treatment shows diminution and agglutination of cocci. Animal left head down for 30 minutes.

90 hours—temperature 102.7°; irritable but otherwise not notably worse. Cistern and lumbar tap. Cisternal fluid, yellow, purulent; contains about 150 cells per field; cocci markedly decreased and phagocytized. Culture: confluent growth. Lavage lumbar to cistern with 15 cc. serum. Rapid ether recovery.

114 hours—temperature 100.9°; weakness of hind legs; irritable; cistern and lumbar punctures; fluid nearly clear. Second trephine done.

Smears: lumbar—rare cells; no organisms seen. Cistern—rare cells; rare agglutinated pneumococci.

Cultures: fair growth.

Injected 2 cc. serum *via* each frontal trephine. 6 cc. lumbar subarachnoid space. 4 cc. cisterna.

Since sterilization was incomplete after several treatments, it was decided to test out the efficacy of ethylhydrocupreine hydrochloride (optochin) intrathecally. This effect is apparent in the continued protocol.

138 hours—temperature 99.4°. Unable to stand; lethargic. Lumbar and cistern punctures under ether. Cisternal smear: cells 5–10 per o. i. f.; cocci 15–20. Lavage with 15 cc., 0.0002 per cent ethylhydrocupreine hydrochloride in saline. Normal recovery.

168 hours—temperature 102°. Lumbar and cistern punctures under ether; cisternal fluid purulent; cocci very numerous. Injected 6 cc. 0.02 per cent optochin.

186 hours—usual double puncture; fluid contains hundreds of pus cells per field; cocci are diminished and markedly phagocyted. Injected 8 cc. of mixture of 15 cc. 0.2 per cent optochin and 5 cc. serum. Breathing ceased but was immediately renewed upon giving artificial respiration. Culture: rare colonies.

210 hours—dead. Autopsy: Moderate exudate over cord; brain congested; easily broken, fibrinous adhesions about cistern and over convexity; moderate seropurulent exudate at base and in both lateral ventricles; the latter are considerably dilated (Fig. 4). A small puncture wound in the floor of the fourth ventricle, just above calamus, with small hemorrhage extending into the central canal of the cord; this was the probable cause of death and is the inevitable result of frequent successive punctures into a dangerous region where working space is small. Smears from all regions show pneumococci—scanty in the cord, moderate in cistern and over convexity, fairly abundant at base and in lateral ventricles. The cellular reaction parallels the pneumococcus distribution in intensity. The principal feature in all smears is the extraordinary amount of phagocytosis and destruction of pneumococci. Many cells are loaded with bacterial débris (Fig. 5). Cultures: all regions positive; heart blood: no growth.

Summary: A mild initial infection; first treatment incomplete; considerable delay in instituting subsequent treatments; the latter usually not complete. Finally recourse to optochin in low concentration, resulting in exacerbation in growth of organisms. Gradual tendency toward sterilization with higher drug concentrations; course interrupted by traumatic death. A total of seven treatments, usually only partial in distribution.

As will be seen from the protocol, the initial growth of organisms in the spinal fluid was described as mild. We have made certain observations as to the rate of increase of these mild initial growths; for example, the fluid in one dog infected with  $4\frac{1}{2}$  million pneumococci is described as follows: 19 hours, 7 diplococci found in the entire smear; 23 hours, an average of 10 per oil immersion field; 42 hours, upwards of 100 cocci per field. The speed of progress of the infection becomes very evident and the significance of even a delay of a few hours in instituting treatment is obvious.

One dog afforded opportunity to compare the difference in numbers of pneumococci in washed and unwashed areas of cortex after a single large lavage, followed by optochin.

*Dog 17.*—Fox terrier, female; weight 5 kilos. March 30, 1927, 4.40 p.m., ether; cistern puncture; clear fluid. Injected 5,200,000 Type I pneumococci (6 hour dextrose broth culture; small transplant; growth 260 million per cc.). Good recovery.

24 hours—temperature  $100.7^{\circ}$ ; in bad condition; irritable; unable to stand; convulsive. Morphine; ether; combined cistern and lumbar punctures; fluid cloudy; cells fairly numerous; hundreds of cocci per field. Lavaged from lumbar to cistern 50 cc. warm saline followed by 7 cc. 0.2 per cent optochin. On the 7th cc., respiration ceased, pulse continued strong, but short period of artificial respiration failed to revive; a drug death. Autopsy showed the cord, cistern, and cerebellum covered with a moderate film of exudate; a small triangular area of cortex just above the cerebellum was similar in appearance, but the base and balance of the cortex, where no lavage had penetrated, were overlaid with thick yellow pus and myriads of cocci. There was moderate bacteremia. Figs. 6 and 7 are offered for comparison of smears from washed and unwashed cortical areas. We are, of course, assuming that the initial distribution of cocci was the same.

This animal died from the effect of the drug on the respiratory center, and the manner of death typifies that which we have regularly seen with overdoses of drug. Respiration suddenly becomes shallow and slow, but pulse continues strong until some time after breathing has ceased. This effect on the respiratory center is transitory, and if artificial respiration is vigorously applied, with, if necessary, the addition of intracardiac adrenalin, it is almost always possible to revive the dog. When large concentrations of drug were used, respiratory difficulties were common, but deaths were few; artificial respiration was in two instances maintained for 20 minutes, during which time the

pulse ceased, only to be renewed by adrenalin injections. Recovery ensued.

A word may be said about the drug concentration which has seemed safe. This has been found not to exceed that present in a mixture of 15 cc. of antiserum and 0.75 cc. of 1 per cent optochin. When no antiserum is used, this dose is too high; a safe maximum has not been determined for drug not diluted with serum. If much pus is present, an animal may withstand higher concentrations of drug than would otherwise be the case; if lavage is very effective and the return fluid practically clear, the drug concentration should never exceed that given above. Great caution should be observed if the fluid return through the cistern needle is blocked. With excellent drainage, the drug effect on the respiratory center appears a function of percentage concentration of optochin in the lavaging fluid, rather than one of total cc. of mixture lavaged through the meninges.

Having determined the dosages of optochin-serum mixture which were tolerated with little or no respiratory disturbance, systematic treatments were again undertaken. The results are evident from the following representative protocols. For convenience, we are illustrating the type of result obtained in mild initial infections and comparing these with that secured in severe initial infections.

*Dog 25.*—Female hound; weight 7½ kilos. April 25, 1927, 3.45 p.m., ether; cistern puncture; clear fluid. Injected 10,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 800 million per cc.). Good recovery.

19 hours—temperature 103.4°. Sluggish; has eaten. Morphine; ether; lumbar and cistern punctures; fluid cloudy; contains 15–20 cells per o. i. f., and about 1 diplococcus to every 3 fields; lavage attempted, but fluid came through with great difficulty and was blood-tinged. Lavage consequently abandoned. After drainage a mixture of serum 10 cc. and 1 per cent optochin 0.5 cc. was distributed equally between cord and cistern. Double frontal trephines were done and 3 cc. of a similar mixture introduced on each side. Cistern culture, abundant growth.

42 hours—temperature 101.3°. Sluggish; lateral nystagmus (traumatic?). Morphine; ether; double puncture; yellow opalescent fluid; cells 15 per o. i. f.; only 1 coccus seen; culture grew but 2 colonies. Injected 10 cc. saline with poor recovery; followed by 5 cc. optochin-serum mixture in lumbar subarachnoid space, and 2 cc. in each frontal. Good recovery.

66 hours—temperature 99°. No treatment. Subsequent uneventful recovery.

*Dog 26.*—Female hound; weight 5½ kilos; a companion dog to the preceding. Infected with 8,000,000 pneumococci from the same culture.

20 hours—temperature 102.9°. Sluggish. Morphine; ether; quadruple puncture. Cisternal fluid cloudy; cells 15–30 per o. i. f.; cocci average 1 to every 2 fields. Saline lavage lumbar to cistern successful at first, but return then failed; repeated washing and draining *via* cisternal needle. Injection of mixture 15 cc. serum and 1.5 cc. 1 per cent optochin, divided 5 cc. cord, 5 cc. cistern, 2 cc. each frontal. Culture, abundant growth.

42 hours—temperature 102°. Clinically normal; usual double puncture, lumbar and cistern. Fluid clear; contains 2–3 cells per field, about half small lymphocytes. Introduced 6 cc. serum into lumbar subarachnoid space and placed head downward. No drug; no frontal treatment. Culture sterile.

66 hours—temperature 101°. No treatment.

Remained unchanged until the 16th day. Found prostrate and convulsive; snapped and salivated and was thought to have rabies. Chloroformed. Typical pneumococcus meningitis, generalized over convexity and base; cord relatively free. Smears show large numbers of pus cells and diplococci; fairly good phagocytosis. Ventricles dilated; purulent ventriculitis. Type I pneumococci recovered from all regions including even the ethmoid cells.

Summary: Mild initial process; sterilization incomplete; reinfection delayed and from some focus so small that in the period between the first treatment and the cistern tap, 24 hours later, no reinfection of the cisternal fluid had occurred, thereby giving a false negative culture.

The subsequent protocol is illustrative of the importance of residual foci in reinfesting meninges not completely sterilized. Experiments such as this are the type which convince us of the inefficacy of partial lavages as practiced by Kolmer.

*Dog 20.*—Female airedale; weight 9½ kilos. Has canine distemper. April 6, 1927, morphine; ether; cisternal puncture; clear fluid; injected 10,500,000 Type I pneumococci (6 hour dextrose broth culture; growth 1,200,000,000 per cc.). Good recovery.

19 hours—temperature 102.8°; lethargic; morphine; ether; lumbar and cistern punctures; fluids purulent; contain several hundreds of cells and hundreds of pneumococci per o. i. f. (Fig. 8). Essentially no phagocytosis; a very severe infection. Lavage lumbar to cistern with 30 cc. saline followed by 11 cc. of mixture of serum 10 cc. and 1 per cent optochin 0.5 cc. Double frontal trephine with injection of 3 cc. of similar mixture on each side; 10 cc. of 800 unit antibody solution (Felton) intravenously; spinal fluid culture, confluent growth.

24 hours—temperature 100°. Ether; double puncture; lumbar and cistern; fluid clearer; cells 50 per o. i. f.; some contain phagocytosed pneumococci (Fig. 9); practically no free organisms; culture, scattered colonies. Lavage lumbar to cistern with 10 cc. saline followed by 0.035 per cent optochin-serum 7 cc.; no frontal treatment.



43 hours—temperature 98.8°; sluggish but otherwise unchanged; morphine; ether; lumbar and cistern punctures; fluid yellowish, opalescent; cells 10–35 per o. i. f.; no organisms seen. Injected 6 cc. serum, lumbar subarachnoid space and placed head down. 75 cc. intraperitoneal saline. Culture yielded about 300 colonies from a 0.5 cc. planting.

65 hours—temperature 102.7°; quite ill; nose a mass of thick pus; cistern and lumbar punctures; fluid a culture of pneumococci, hundreds per field (Fig. 10); cells rare; no agglutination nor phagocytosis. Represents a massive reinfection of a nearly sterile region, following cessation of complete treatments. Injected 8 cc. 0.05 per cent optochin-serum mixture divided between cord and cistern and 3 cc. into each frontal.

90 hours—temperature 99.6°; weaker; able to stand; ataxic; no change in distemper; ether; cistern and lumbar punctures; no fluid obtained. Gave 5 cc. lumbar, 1 cc. cistern, 3 cc. each frontal of 0.1 per cent optochin-serum. Intraperitoneally 150 cc. 10 per cent glucose; milk by stomach tube.

114 hours—temperature below 94°; chloroformed. Autopsy: Material suggesting chicken fat clot in fourth ventricle, extending down over cervical cord. Explains the last "dry tap." Fibrin at base, about pituitary and optic chiasm. Cord elsewhere relatively clear; anterior convexity relatively clear; meninges over posterior convexity opaque; exudate purulent; some pus in lateral ventricles.

Region	Cells per o. i. f.	Cocci	Culture
Cord (lumbar)	5–10	Rare phagocyted; very rare free	0
Cistern	20–75	Moderate; free and phagocyted	+
Anterior convexity	5–25	Only 2 cocci in entire smear	0
Posterior " (Fig. 11)	75	Rare; phagocyted	+
Base	25	Moderate; free and phagocyted	+
Lateral ventricle (Fig. 12)	75	Moderate, phagocyted; more, free	+

A pneumonia practically lobar in distribution, entire left lung; culture, *bronchi-septicus*. Negative heart's blood.

Summary: Initial massive infection; tendency to sterilization with treatment; "reinfection" with cessation of treatment; renewed tendency toward sterilization with resumption of treatment. Main focus of infection lateral ventricle; course of disease interrupted by fatal outcome of distemper.

As examples of severe infections sterilized slowly and progressively by successive complete treatments in one instance, seven in all, necessitating altogether ten etherizations, partially for treatment and partially for diagnosis, the following may be offered.

*Dog 29.*—Male hound; weight 7½ kilos. May 2, 1927, 4.00 p.m., morphine; ether; cistern puncture. Clear fluid. Injected 10,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 680 million per cc.). Good recovery.

24 hours—temperature 102.2°. Sluggish; lumbar puncture; fluid under increased tension, opalescent, yellowish; cells 50 per o. i. f.; diplococci 5–12 per field. Not treated. Culture, confluent growth.

42 hours—temperature 103.7°. Sluggish but able to stand. Morphine; ether; quadruple puncture; lumbar fluid yellowish, opalescent; cells 8–10 per o. i. f.; diplococci about 100 per field; similar cisternal fluid (Fig. 13). Lavage attempted, but proceeded with difficulty and with poor return through cisternal needle; abandoned. Injected 7 cc. of mixture of serum 10 cc., optochin 1 per cent, 0.5 cc. cisterna, 3 cc. lumbar, and 2 cc. similar mixture in each frontal subarachnoid region. Cultures, confluent growth.

66 hours—temperature 102.9°. Clinically unchanged. Morphine; ether; quadruple puncture. Cisternal fluid clearing; cells rare; diplococci less than 20 per field. Lavaged lumbar to cistern with 10 cc. of saline; injected serum-optochin mixture as above. Gave 100 cc. intraperitoneal glucose. Culture, confluent growth.

90 hours—temperature 102.8°. Unchanged. Quadruple puncture; fluid more cloudy; cells 20–40 per field; cocci double in number. Last treatment repeated. No lavage. Culture, confluent growth.

96 hours—treatment repeated with exception of frontal injections. Cisternal fluid cloudier; cells increased but what few pneumococci are present are all phagocyted. Culture, few colonies.

114 hours—temperature 102.6°. Clinically unchanged. Morphine; ether; cisternal puncture; fluid scanty; 6–7 diplococci per field; culture, colonies increased. Complete treatment as above with exception of left frontal; no lavage.

138 hours—temperature 104°; unchanged; has eaten and drunk; stands and wags tail. Ether; quadruple puncture; lavage lumbar to cistern with 15 cc. serum-optochin mixture (serum 15 cc., optochin 0.75 cc. of 1 per cent solution); 1.5 cc. same mixture, both frontal trephines. Fluid clearing; cells 5–10 per field; no cocci seen. Culture, scanty growth.

162 hours—temperature 103.7°; canine distemper; less active; will not stand without assistance. Ether; lumbar and cistern punctures; cells 4–5 per field; no cocci; culture, sterile; lavage with 14 cc. serum-optochin mixture as above. Intraperitoneal glucose, 100 cc.

186 hours—temperature 102.7°; distemper worse; thick pus flowing from nostrils. No treatment.

210 hours—unchanged; diagnostic cistern puncture; rare cells; no cocci; injected 5 cc. of serum as prophylactic; culture, negative.

288 hours—very ill from distemper. Subnormal temperature. Chloroformed. Bilateral bronchopneumonia (*B. bronchisepticus*). Entire brain and cord grossly normal save for slight excess of small lymphocytes and endothelial leucocytes in

cisternal and ventricular fluids. Cultures taken from all regions, including the entire fluid contents of the lateral ventricles, failed to give growth.

Microscopically some slight generalized infiltration of the meninges by endothelial leucocytes, many fatty, a few with blood pigment; a typical late clearing up stage. In addition, collections of lymphocytes in the region just beneath the ependyma of ventricles. An organizing (purpuric?) hemorrhage of gray matter of cord.

*Dog 32.*—Female hound; weight 5 kilos. May 4, 1927, 4.05 p.m., morphine; ether; cistern puncture; clear fluid. Injected 7,500,000 Type I pneumococci (6 hour dextrose broth culture; growth 750 million per cc.). Good recovery.

22 hours—temperature 104°; sluggish; irritable. Ether; lumbar puncture; fluid seropurulent; cells 15–20 per o. i. f.; cocci upwards of 150 per field (Fig. 14); cisternal fluid opalescent; cells rare; cocci 20–30 per field. Lavage, lumbar to cistern with 15 cc. saline. Frontal trephines; injection of mixture of 15 cc. anti-serum and 0.75 cc. 1 per cent optochin as follows: lumbar subarachnoid space, 7 cc., cisterna magna, 3 cc., each frontal trephine, 2 cc. Culture. confluent growth.

42 hours—temperature 102.4°; stands weakly; attempts to walk; drinks. Ether; quadruple puncture; cisternal fluid clearing; cells 20–30 per field; no cocci seen; culture, colonies numerous but growth not confluent; injected serum-optochin mixture (as above) 7 cc. lumbar, 3 cc. cistern, 1 cc. each frontal.

66 hours—temperature 101°; marked loss of weight; weakness hind legs. Ether; quadruple puncture; fluid almost clear; cells 2–3 per field and cocci about 1 in every 2 or 3 fields; culture, colonies increased. Treatment as at 42 hours. Intraperitoneal glucose.

90 hours—temperature 98°; definite partial paraplegia hind legs; probably traumatic. No treatment.

114 hours—temperature 98.6°; unchanged; diagnostic cistern puncture; abundant crystal clear fluid; lymphocytes less than 1 per field; no organisms. Plated 2 cc. of fluid; no growth.

138 hours—temperature 99.3°; diagnostic puncture; negative culture. Subsequent course uneventful; persistent partial paraplegia.

The following may be offered as an example of a case where two complete treatments failed to sterilize the meninges appreciably. The condition was complicated by distemper.

*Dog 34.*—Young female collie pup. May 10, 1927, 4.00 p.m., ether; cisternal puncture; clear fluid. Injected 9,000,000 Type I pneumococci (6 hour dextrose broth culture; growth 610 million per cc.). Good recovery.

19 hours—temperature 104°; sluggish; has developed severe distemper.

23 hours—ether; quadruple puncture; cistern and lumbar fluids seropurulent; cells 50–75 per field; diplococci very numerous. Lavage lumbar to cistern with

20 cc. saline, followed by lavage with serum-optochin mixture (serum 15 cc., 1 per cent optochin 0.75 cc.), lumbar to cistern 15 cc.; frontal trephines 2 cc. each of mixture of similar proportions. Intraperitoneal glucose, 100 cc. Culture, confluent growth.

42 hours—temperature 101.5°; unchanged. Quadruple puncture; complete treatment as above; 100 cc. glucose intraperitoneally. Smear: an 80 per cent reduction in organisms; cells average 12 per field. Culture, confluent growth.

66 hours—temperature below 94°. Chloroformed. In gross the brain and cord show only a trace of exudate; microscopically smears contain relatively few cells; diplococci present in large numbers and save in the cord region, where there is some phagocytosis, no evidence is seen of beneficial effect of treatment. Marked purulent bronchitis, but death is undoubtedly meningeal. Cultures all give confluent growth; heart's blood, rare colonies. Histologically severe, diffuse leptomeningitis.

### *Comments.*

Since optochin is somewhat under suspicion in view of its apparent tendency to produce transient amblyopia, we have endeavored to detect visual disturbances in recovered dogs. So far as was ascertained, none occurred; no ophthalmoscopic examinations were made. Some of the recovered dogs are deaf, but we know that in meningococcal meningitis permanent deafness may result.

A word may be said about lateral ventricular punctures. This has not been done in the dog; it is fairly certain that sterilization would be hastened and that more recoveries would have resulted had this been a routine procedure. Whereas the lateral ventricle of a dog, in the region where puncture is desirable, is a mere slit unless pathologic dilatation has occurred, it should offer no great problem in man. It is apparently the most difficult region to reach with serum by the methods applied in this study. In this respect the base in the region of the chiasm shares with the lateral ventricle and in case these methods should ever be employed in treating human disease, failure to sterilize by the quadruple puncture route should make one think seriously of ventricular punctures and punctures through to the base. The important thing in securing a cure is contact of all regions with the optochin-serum mixture—contact complete and frequently repeated. Undoubtedly lavage is a valuable adjuvant, but lavage alone cannot rid the meninges of organisms and if even a very small number remains, our experience shows that severe reinfection is almost inevitable. The object of treatment should be to wash out as many

organisms as possible and to control the residual bacteria by a pneumococcal drug and a phagocytosis-producing antiserum.

One of the most hopeful and surprising things observed in the entire study was the manner in which lavage may be accomplished even in the presence of a massive exudate, it being possible to lavage a cord in an animal having almost frankly purulent cisternal and lumbar fluids, until the return fluid is nearly crystal clear; full cognizance is, however, taken of the fact that no matter how long one washes, within reason, cells and pneumococci blocked in fibrin meshes, the former certainly in large numbers, will remain. It has seemed that it is easier to lavage out organisms than it is cells, since the latter are larger and the network of fibrin offers more resistance to their free passage. As judged by the pictures of phagocytosis in foci fairly distant from the site of injection, the immune serum penetrates the exudate very satisfactorily.

No statistics of cures are presented. In view of the prevalence of traumatic deaths after frequently repeated cistern punctures, drug deaths, when high optochin concentrations were used, and especially mortality from epidemic canine distemper, such statistics would be meaningless. The fact that progressive sterilization of the meninges by methods employed in this study is possible has been established in our opinion without question.

### *Pathology.*

The microscopic pathology has been studied in all animals dying of the disease, and in certain recovered dogs which succumbed to intercurrent disease (distemper) after becoming sterile. Sections show a rapidly spreading, fibrinopurulent leptomeningitis, which as early as 23 hours after infection may involve all regions of the meninges (Fig. 15). In a control dog, or in a treated animal in which the disease was not checked, the exudate reaches large proportions; the cord dura is invaded; the spinal nerve roots are involved (Fig. 16); there is an inflammatory process in the epidural fat (Fig. 17). Invasion and destruction of the choroid plexuses, superficial encephalitis, spread *via* the Virchow-Robin spaces are common (Figs. 18-20). An empyema of the third or fourth ventricle with extension into the central canal of the cord may occur (Fig. 21). Probably, too, the latter may

be reached from the ventral fissure. Once in the central canal the process may destroy the ependyma and invade the region of the commissures, and from thence the cornua by following a perivascular route, giving rise to a myelitis. Invasion, at least to any great extent, of the lateral ventricles occurs relatively late, probably because of the direction of flow of the spinal fluid; in other words, the lateral ventricles lavage themselves. With the development of a blocking exudate over the convexity or in the fourth ventricle or aqueduct, empyemas with dilatation of the lateral ventricles inevitably result. With the subsiding of the process, polymorphonuclears disappear and are replaced by fatty and phagocytic endothelial leucocytes and small lymphocytes, which cells apparently may persist for some time. Such was the picture observed in Dog 29, Fig. 22 (see protocol). Slight subependymal and peripheral cortical gliosis may be found.

#### *Reinfection Experiments.*

Five recovered dogs, together with a control, were reinfected to see if any immunity to meningeal infection existed. These animals (Dogs 1-5) were reinoculated 103, 79, 63, 47, and 42 days respectively after primary infection. Four ran an atypical course. The first two developed a very high grade, early leucocytosis; in one of these no organisms were observed in the 24 hour spinal fluid smear, and a culture gave no growth. Dog 1 died of severe purulent meningitis in 114 hours. Dog 2, whose culture was sterile 24 hours after infection, showed organisms at 66 hours; the number of cocci in the smears increased very slowly and at 7 days they were no more numerous than frequently seen after the 1st day in a primarily infected animal. As organisms increased, leucocytes diminished; phagocytosis was rarely seen and can scarcely account for the slow development of the disease. In none of the dogs were agglutinins demonstrable in the spinal fluids. Dog 2 died the 9th day. Dogs 3 and 5 gave negative cultures, and smears contained but 20-30 leucocytes per field 24 hours after infection; by 66 hours fluids were crystal clear and sterile. Dog 4 ran a typical course, but was sterilized by two complete optochin-serum treatments. This sterilization was unusually rapid. The control dog died typically in 48 hours. Hence a degree of increased resistance remained within the meninges.

## RÉSUMÉ AND CONCLUSIONS.

1. Extensive acute, fibrinopurulent meningitis may be produced in dogs by the intracisternal injection of virulent Type I pneumococci.

2. Given an equal number of virulent infecting organisms, the rate of establishment of infection depends upon the phase of growth and the quantitative growth per cc. of culture plus an uncontrollable individual factor in the animal.

3. The pathology of pneumococcal meningitis is discussed. It resembles very closely the similar disease in man.

4. Systematic lavage and treatment with optochin-serum mixtures by the method of quadruple puncture, as described above, have resulted in cures of Type I pneumococcal meningitis in dogs.

5. The important factor in obtaining cures is to bring all regions of the meninges into frequent contact with the therapeutic agent.

6. In the absence of such contact, incomplete sterilization results and "reinfection" is almost inevitable.

7. Protocols show the necessity of repeated negative cisternal fluids, both on smear and on culture, before sterilization can be assured.

8. Recovered dogs subjected to meningeal reinfection show some degree of resistance.

## BIBLIOGRAPHY.

1. Stewart, F. W., *J. Exp. Med.*, 1927, xlv, 391.
2. Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 373.
3. Kolmer, J. A., *Arch. Otolaryngol.*, 1926, iii, 481.
4. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

## EXPLANATION OF PLATES.

## PLATE 16.

FIG. 1. Control Dog 3. Massive basal exudate.

FIG. 2. Control Dog 7. Smear from convexity.  $\times 1000$ .

FIG. 3. Distribution of dye introduced *via* left frontal trephine.

## PLATE 17.

FIG. 4. Treated Dog 10. Dilated ventricles; purulent ventriculitis.

FIG. 5. Treated Dog 10. Ventricular smear showing extensive phagocytosis after ethylhydrocupreine hydrochloride treatment.  $\times 1000$ .

FIG. 6. Treated Dog 17. Smear from posterior (lavaged) cortex.  $\times 1000$ .

## PLATE 18.

FIG. 7. Treated Dog 17. Smear from anterior cortex unaffected by lavage and chemo-serotherapy.  $\times 1000$ .

FIG. 8. Treated Dog 20. Initial 19 hour cisternal smear.  $\times 1000$ .

FIG. 9. Same dog. 24 hour smear (after one treatment).  $\times 1000$ .

FIG. 10. Same dog. 65 hour smear, showing result of cessation of treatment.  $\times 1000$ .

## PLATE 19.

FIG. 11. Same dog. Smear from posterior convexity taken at 114 hours. Rare phagocytosed cocci.  $\times 1000$ .

FIG. 12. Same dog. Lateral ventricle smear.  $\times 1000$ . Lateral ventricle a residual focus of infection.

FIG. 13. Recovered Dog 29. Cisternal smear before treatment.  $\times 1000$ .

FIG. 14. Recovered Dog 32. Cisternal smear before treatment.  $\times 1000$ .

## PLATE 20.

FIG. 15. Control dog, showing extent of exudate at 23 hours.  $\times 50$ .

FIG. 16. Control dog. Massive exudate. Invasion of spinal nerve.  $\times 50$ .

FIG. 17. Treated dog. Accidental death. Inflammatory exudate in epidural fat.  $\times 50$ .

FIG. 18. Control dog. Purulent ventriculitis; superficial encephalitis; destruction of choroid plexus of lateral ventricle.  $\times 50$ .

## PLATE 21.

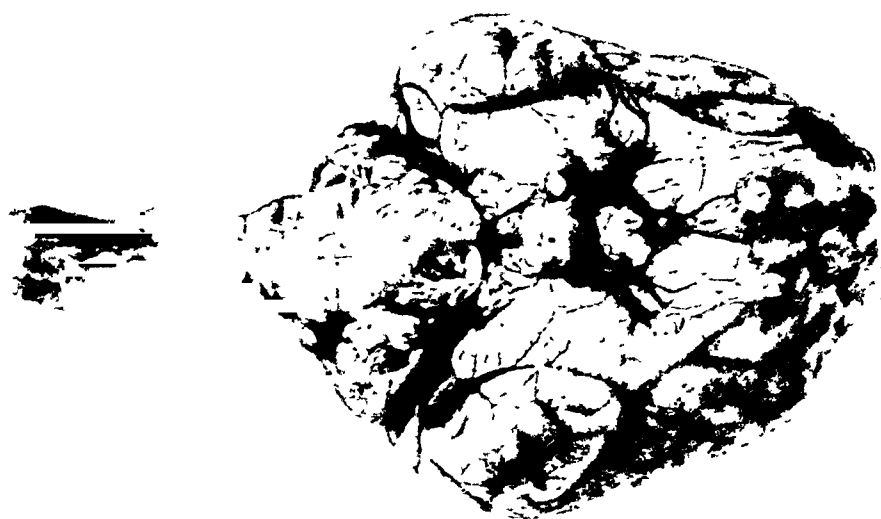
FIGS. 19 and 20. Control dog. Perivascular infiltration of cortex.  $\times 50$ .

FIG. 21. Control dog. Empyema of central canal of cord; myelitis.  $\times 50$ .

FIG. 22. Treated Dog 29. Sterile cultures; death from distemper; meninges essentially negative.  $\times 50$ .



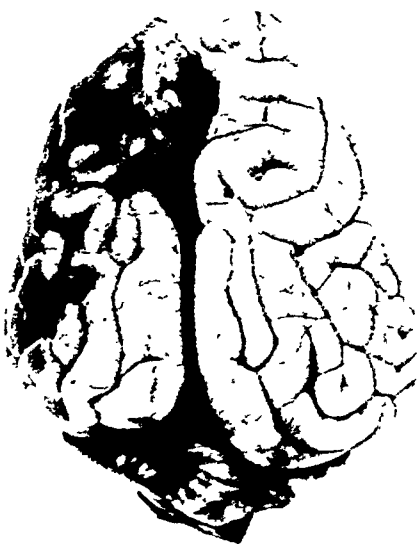




1

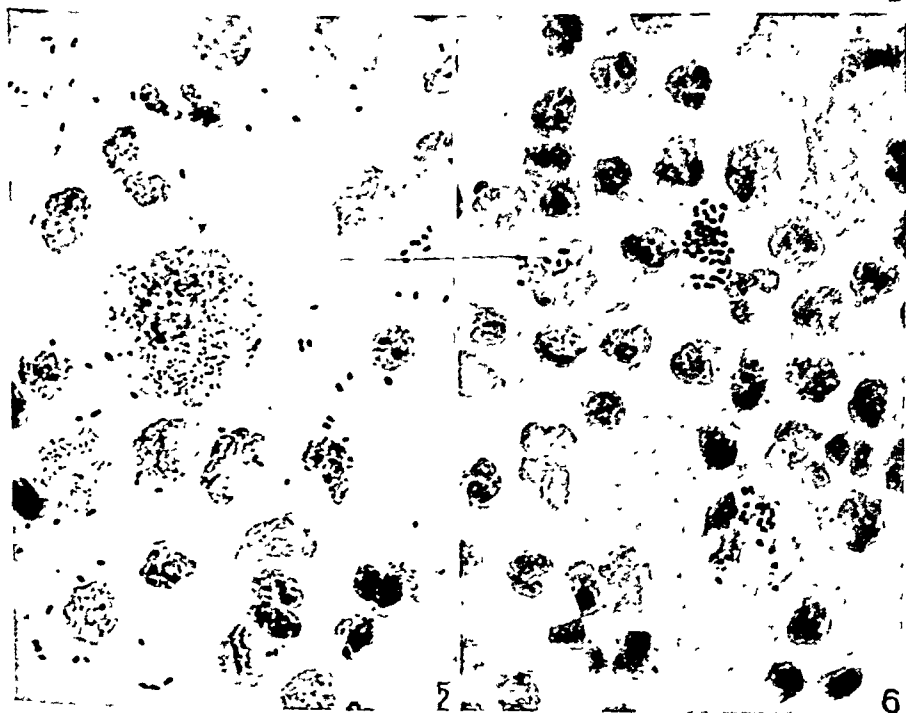


2

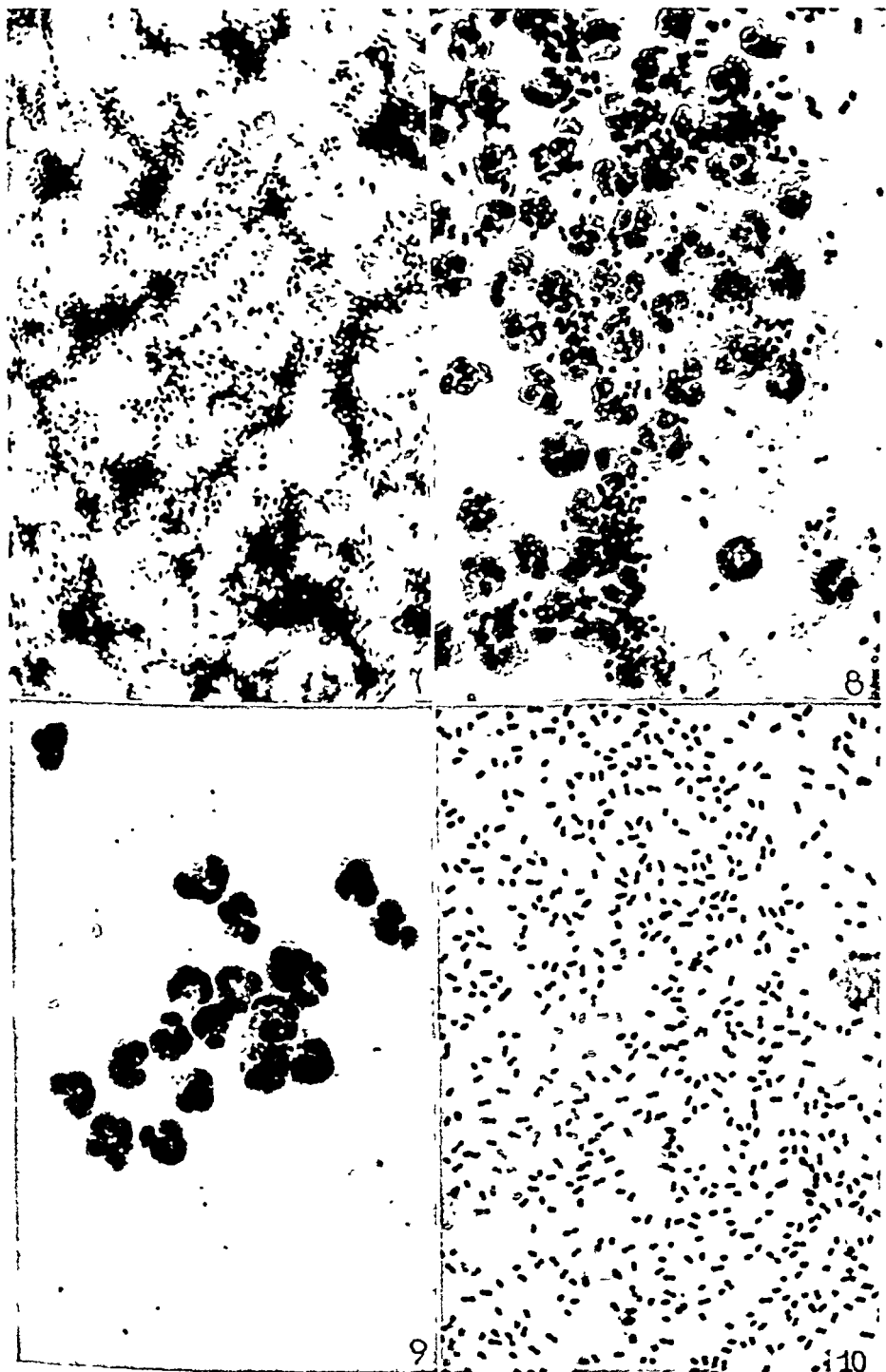


3

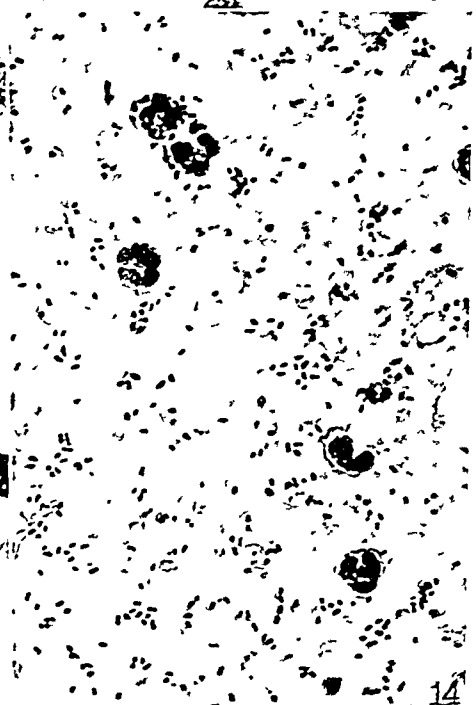
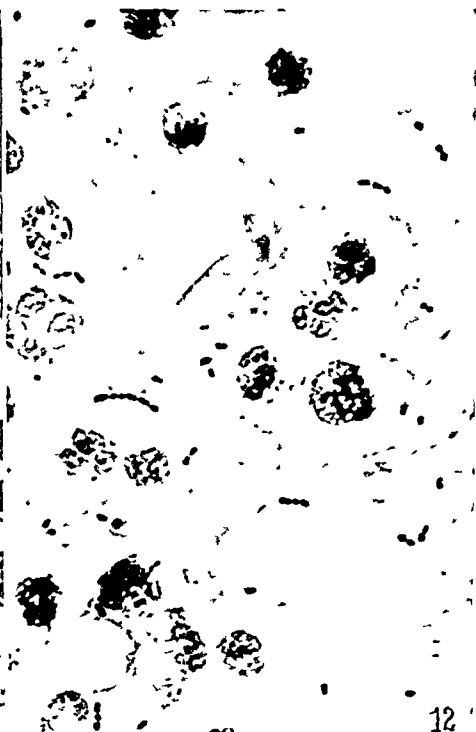
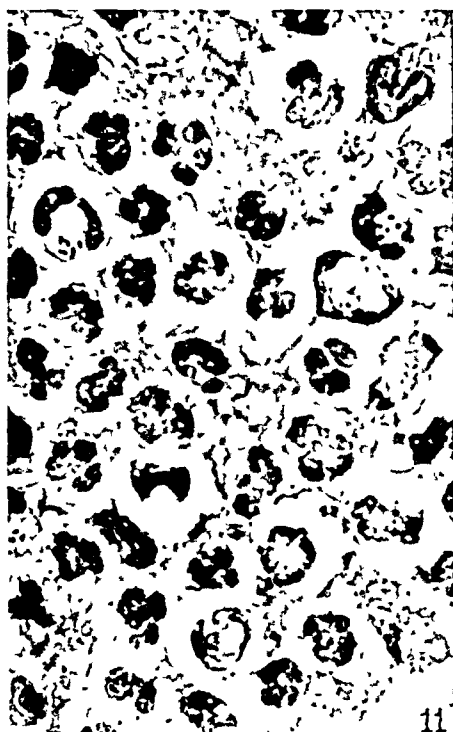
























# ENLARGEMENT OF THE ADRENAL CORTEX IN EXPERIMENTAL UREMIA.\*

By EATON M. MACKAY, M.D., AND LOIS LOCKARD MACKAY, Ph.D.

(From the Department of Medicine, Stanford University Medical School,  
San Francisco.)

PLATES 22 AND 23.

(Received for publication, June 10, 1927.)

The adrenal glands of a number of individuals who died in the uremia<sup>1</sup> of terminal inflammatory Bright's disease have been found to be larger than those obtained at postmortem following death from other causes. These patients usually if not always suffer from secondary infections and infections of various kinds have been found (1, 2) to cause enlargement of the adrenal glands. It is therefore difficult to say whether the adrenal enlargement is a result of the uremia *per se* or is brought about by secondary infections. The experiments which are described here were accordingly carried out to determine the effect in the rat of an uncomplicated experimental uremia on the weight and size of the adrenals.

## Methods.

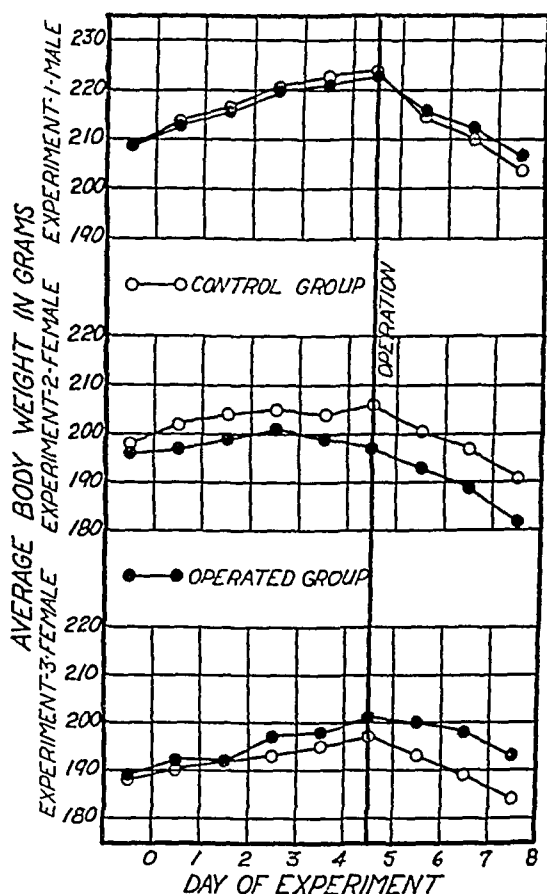
Albino rats were used as the experimental animals. Because of the gross difference in the weights of the adrenal glands of the two sexes (3) a separate experiment was carried out on each sex. All the animals in an experiment were of the same sex and of exactly the same age. In each experiment the rats were arranged in order of their body weight, every alternate animal being placed in the control group. Both groups were put on a special diet and weighed daily for 5 days before operation. On the day of operation both kidneys were removed from each

---

\* This work was aided by the Wellington Gregg Fund for the Investigation of Bright's Disease.

<sup>1</sup> The use of the term "uremia" is limited here to the cases having an abnormal retention of non-protein nitrogen in the blood and does not include patients suffering from so called uremic symptoms whose blood urea concentrations were not abnormal.

rat of the operated group through short lumbar incisions while the animal was under ether anesthesia. Within a very few minutes following the removal of both organs and the suspension of the anesthetic the animal was moving about its cage. The rats in the control group were treated in exactly the same manner as those undergoing the double nephrectomy except that their kidneys were only exposed. From the time of operation until they were killed the animals of both groups were without food but allowed water.



TEXT-FIG. 1.

The rats of the operated group were just as lively as those in the control group until about 70 hours after operation when the former would become comatose and show muscular twitchings. In order to avoid this terminal stage both groups were sacrificed about 60 hours after operation. Each rat was weighed and then anesthetized with ether. A blood specimen was obtained from the abdominal aorta and its urea content determined later. The adrenals were then removed,

carefully freed from adherent fat and connective tissue and weighed. This weight was recorded as the "wet" weight. The tissue was then fixed in 10 per cent neutral formalin.

The adrenals in each group were arranged in order of the magnitude of the adrenal weight, body weight, ratio. Every other pair of glands was imbedded in paraffin and sectioned serially. One 10 micron section from every 50 micra of tissue was preserved for determining cortex or medulla volume changes. The other half of the fixed adrenals of each group was washed 24 hours in distilled water and their "dry" weight determined after dehydration in a vacuum oven at 70°C. In one experiment the defatted dry weights were determined by extracting the dehydrated glands with hot anhydrous ether, an alcohol-ether mixture and finally absolute alcohol, several times in each case. The extracted glands were then further dried under reduced pressure and weighed.

Experiment 1 was performed with male rats 100 days of age at operation while in Experiment 2 female rats 180 days of age at the same time were used. The body weights for the two experiments are charted in Text-fig. 1. Neither the control nor operated groups lost very much weight during the 3 days that they were without food. Since in neither Experiment 1 nor 2 were the dried adrenals preserved for further treatment a third experiment was performed in order to obtain "defatted" adrenal weights. In this experiment female rats 170 days of age at the time of operation were used.

### *Results.*

The results of Experiments 1 and 2 are given in Tables I and II. These give indisputable evidence of adrenal enlargement following the production of an experimental uremia by means of bilateral nephrectomy. Except for the inherent sex difference in the weight of the adrenals the results are essentially the same for both sexes. The actual differences between the averages of the fresh or "wet" adrenal weights of the two control and operated (uremic) groups are, for the males, 10.9 mg. and, for the females, 13.6 mg. while the probable difference of the average determined by the usual method is only  $\pm 0.72$  mg. for the males and  $\pm 1.71$  mg. for the females. The degree of adrenal enlargement in these experiments as determined by the "wet" weight of the glands 60 hours after the removal of both kidneys from the operated group amounted to 64.9 per cent in the male experiment and 46.8 per cent in the case of the female experiment.



Since the average initial body weights of the control and operated groups are practically alike the results (Tables I and II) determined from actual weights and adrenal weight in relation to the initial body weight are essentially the same. The latter only are considered here.

TABLE I.  
*Experiment 1 (Males).*

No.	Body weight		Blood urea concentration	Adrenal weight	Adrenal tissue per 100 gm. body weight	
	Original	Final			"Wet"	"Dry"
	gm.	gm.			mg. per 100 cc.	mg.
Control group						
1	190	179	30.8	36.1	18.9	—
3	216	210	26.3	40.0	18.5	6.4
5	190	180	30.9	35.0	18.4	—
7	204	194	27.3	37.0	18.1	6.8
9	200	196	32.4	35.3	17.5	—
11	208	202	30.0	35.8	17.3	5.8
13	210	216	27.2	35.0	16.7	—
15	226	221	31.7	36.6	16.4	5.6
17	214	206	28.1	29.0	13.5	—
19	230	231	33.6	30.2	13.0	5.0
Mean	209	204	29.8	35.0	16.8	5.9
Operated (uremic) group						
2	204	204	550.0	68.0	33.3	—
4	212	219	574.6	68.1	32.1	10.5
6	198	198	556.8	56.9	28.8	—
8	220	216	551.0	61.7	28.2	11.2
10	180	173	700.0	50.0	27.8	—
12	196	193	550.7	51.6	26.5	10.1
14	206	208	566.0	54.2	26.2	—
16	224	220	487.5	56.0	25.0	8.1
18	238	240	570.0	59.1	24.7	—
20	212	204	549.0	52.0	24.5	8.6
Mean	209	207	565.6	57.8	27.7	9.7

It is conceivable that the enlargement of the adrenals which ensued in the uremic animals is a result not of hypertrophy but simply of an increase in weight brought about by an edema or at least an increase in water content. In each alternate animal in Experiments 1 and 2

(Tables I and II) and in every case in Experiment 3 (Table III) the glands were dehydrated by the methods which have been described and the "dry" weight of adrenal tissue determined. In Experiment 1 (males) the actual difference between the averages of the "dry"

TABLE II.  
*Experiment 2 (Females).*

No.	Body weight		Blood urea concentration	Adrenal weight	Adrenal tissue per 100 gm. body weight	
	Original	Final			"Wet"	"Dry"
	grs.	grs.			mg. per 100 cc.	mg.
Control group						
1	172	166	36.4	63.0	36.6	—
3	170	164	25.5	56.8	33.5	12.8
5	218	210	31.7	69.1	31.6	—
7	200	194	21.8	63.2	31.5	12.5
9	180	180	32.7	53.0	29.4	—
11	200	195	27.3	56.3	28.0	9.8
13	189	182	35.4	51.0	27.0	—
15	226	220	31.2	60.0	26.5	6.2
17	212	198	24.5	55.0	25.9	—
19	212	200	30.8	43.2	20.3	6.8
Mean	198	191	29.7	57.1	29.0	9.6
Operated (uremic) group						
2	164	154	743.2	95.0	58.0	—
4	200	186	650.0	98.1	49.0	13.6
6	175	165	760.0	78.2	44.5	—
8	192	182	712.5	83.4	43.3	13.6
10	190	180	658.2	80.8	42.6	—
12	216	200	550.0	90.3	41.7	12.8
14	226	212	698.1	91.1	40.3	—
16	212	196	572.0	81.4	38.2	12.0
18	175	166	745.5	64.0	36.6	—
20	206	180	702.0	66.0	32.0	10.9
Mean	196	182	679.2	82.8	42.6	12.6

adrenal weights of the control and operated groups is 3.8 mg. while the probable difference is  $\pm 0.40$  mg. For Experiment 2 (females) the same figures are 3.0 mg. and  $\pm 0.89$  mg. and in Experiment 3 (females) 3.3 mg. and  $\pm 0.59$  mg. respectively. The degree of ad-

renal enlargement determined from the dry tissue weights in the three instances is 64.6 per cent (Experiment 1), 31.3 per cent (Experiment 2) and 32.7 per cent (Experiment 3). A large increase in weight is still present so that it is safe to conclude that an increased water content is not the sole cause of the heavy adrenals in the uremic rats. However since in neither case is the degree of enlargement as great as that found for the wet tissue weights the increase in water content was of somewhat greater magnitude than the degree of increase in solids. The increase in solids in the three experiments was 64.6 per cent, 31.3 per cent and 32.7 per cent respectively while the increase in water amounted to 65.1 per cent, 54.6 per cent and 77.3 per cent respectively. In one case (Experiment 1, males) the increases in solids and water content were the same.

Landau and McNee (4) have determined chemically the content of cholesterol and cholesterol esters in the adrenals for a number of conditions. Among these were several uremic individuals whose adrenal glands contained a higher percentage of cholesterol and cholesterol esters than any of the other cases examined. Because of this, and the interest which has often been manifested in the lipid content of the adrenals in relation to changes in these glands, in one experiment (Experiment 3) the glands were defatted with appropriate solvents to determine the possible increase in lipid content as a cause of the increased weight. The results in Table III indicate that substances soluble in fat solvents contributed more to the weight of the uremic than of the control adrenals, for the degree of hypertrophy determined from the dried defatted glands was less: 21.0 per cent as compared with the 32.7 and 31.3 per cent found for the dried glands which were not defatted.

As we have shown elsewhere (5) the compensatory hypertrophy of the remaining gland which follows unilateral adrenalectomy is limited to the cortex. The adrenal enlargement which follows chronic intoxication as in chronic infections (1) and after repeated injections of foreign proteins (2) in the production of antisera also appears to be confined to the cortex. In the present experiment likewise the hypertrophy was limited to the cortex. Alternate glands from Experiments 1 and 2 were available for histological examination. After fixing these were imbedded in paraffin and serial sections made.

TABLE III.  
*Experiment 3 (Females).*

No.	Body weight		Adrenal weight	Adrenal tissue per 100 gm. body weight		
	Original	Final		"Wet"	"Dry"	"Defatted"
	gms.	gms.		mg.	mg.	mg.
Control group						
1	170	172	68.3	40.2	14.7	10.8
3	174	174	59.8	34.4	12.9	11.8
5	164	162	49.8	30.3	7.6	5.5
7	194	176	57.7	29.7	9.3	9.2
9	168	168	47.0	28.0	9.1	6.0
11	204	184	57.0	27.9	10.8	10.1
13	180	182	49.2	27.3	9.2	7.8
15	180	182	48.6	27.0	9.7	7.8
17	186	176	50.0	26.9	9.7	9.6
19	206	196	54.6	26.5	9.9	4.8
21	214	210	56.0	26.1	10.2	9.0
23	198	190	48.9	25.2	10.6	8.2
25	200	202	49.9	25.0	9.0	7.7
27	189	190	47.1	24.9	9.3	6.3
29	200	192	48.1	24.0	9.4	7.2
Mean	188	184	52.8	28.2	10.1	8.1
Operated (uremic) group						
2	200	216	112.2	56.1	20.7	11.8
4	169	163	92.3	54.6	17.2	11.8
6	186	180	92.1	49.6	11.2	8.5
8	164	168	81.4	49.6	17.2	10.9
10	178	200	87.3	49.2	12.7	9.0
12	189	194	89.4	47.3	11.5	10.1
14	170	170	80.1	47.1	15.0	10.5
16	199	194	89.9	45.0	13.8	9.7
18	200	203	81.2	40.6	13.3	10.1
20	186	186	75.6	40.6	11.7	9.9
22	186	208	73.0	39.2	11.7	10.0
24	222	222	85.3	38.5	13.0	10.5
26	172	168	65.4	38.0	11.6	8.5
28	200	204	75.2	37.6	12.9	9.2
30	212	224	59.8	28.2	8.1	5.6
Mean	189	193	82.7	44.1	13.4	9.8

Precautions were taken during the imbedding to reduce the shrinkage to a minimum. Whatever reduction in size resulted was however probably of about the same degree in each group. Every fifth 10 micron section was accurately outlined by means of a camera lucida at a magnification of 35 diameters on heavy paper of uniform weight

TABLE IV.  
*Experiment 1 (Males).*

Experiment 1 (Males).

No.	Adrenal volume	Medulla volume	Adrenal volume per 100 gm. original body weight		
			Whole gland	Medulla	Cortex
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
Control group					
1	7.8	0.7	4.10	0.37	3.73
5	5.7	0.5	3.00	0.26	2.74
9	7.7	0.8	3.85	0.40	3.45
13	8.0	0.7	3.81	0.33	3.48
17	5.4	0.4	2.52	0.19	2.33
Mean	6.9	0.6	3.45	0.31	3.15
Operated (uremic) group					
2	8.4	0.5	4.12	0.24	3.88
6	7.3	0.6	3.69	0.30	3.39
10	11.2	0.9	6.22	0.50	5.72
14	9.0	0.6	4.37	0.29	4.08
18	12.7	0.9	5.32	0.38	4.94
Mean	9.7	0.7	4.74	0.37	4.40
			Difference of means		Hypertrophy
			Actual	Probable	
Whole gland.....			1.29	±0.33	<i>per cent</i>
Cortex.....			1.25	±0.30	37.4
Medulla.....			0.06	±0.11	39.7
					(19.3)

per unit area. The medulla if present in the section was also outlined. Typical central sections through the glands of Experiments 1 and 2 are reproduced in Text-fig. 2. The area of these enlarged paper sections was determined by their weight. These areas of the whole gland and of the medulla, based on 10 micron sections, were

TABLE V.  
*Experiment 2 (Females).*

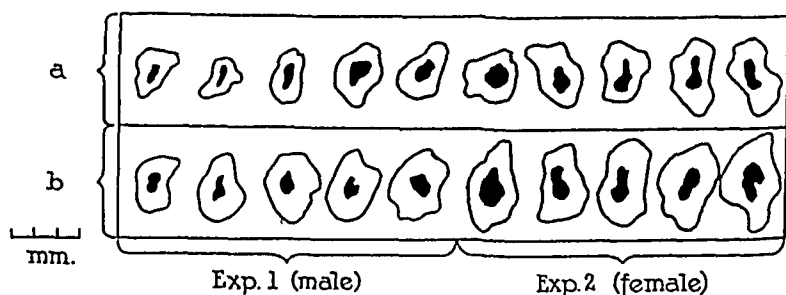
No.	Adrenal volume	Medulla volume	Adrenal volume per 100 gm. original body weight		
			Whole gland	Medulla	Cortex
	c.c.m.m.	c.c.m.m.	c.c.m.m.	c.c.m.m.	c.c.m.m.
Control group					
1	10.3	1.2	6.00	0.70	5.30
5	10.3	0.9	4.73	0.41	4.32
9	9.6	0.7	5.31	0.39	4.92
13	7.3	0.8	3.86	0.42	3.44
17	7.6	0.6	3.58	0.28	3.30
Mean	9.0	0.8	4.69	0.44	4.25
Operated (uremic) group					
2	14.4	1.2	8.78	0.73	8.05
6	13.4	0.6	7.66	0.34	7.32
10	13.1	0.8	6.90	0.42	6.48
14	14.1	0.9	6.22	0.40	5.82
18	12.6	1.1	7.20	0.63	6.57
Mean	13.5	0.9	7.35	0.50	6.85
			Difference of means		Hypertrophy
			Actual	Probable	
					<i>per cent</i>
Whole gland.....			2.66	$\pm 0.36$	56.6
Cortex.....			2.60	$\pm 0.33$	61.2
Medulla.....			0.06	$\pm 0.20$	(13.7)

TABLE VI.  
*Percentage Increase of Adrenals in Uremia.*

	Whole gland					Cortex	Medulla
	Weight	Solids	Water	Lipids	Volume	Volume	Volume
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Males.....	64.9	64.6	65.1		37.4	39.7	19.3
Females.....	46.8	32.0	65.9	80.0	56.6	61.2	13.7

considered representative of the 50 micron slabs from which they came and the volume of the 50 micron section thus determined. The volume of the cortex and medulla of each gland was then determined. These results comprise Tables IV and V.

It is evident from both experiments that the hypertrophy is limited to the cortex as there is no significant difference in the medulla volumes of the control and hypertrophied glands. In the case of the males when considered as hypertrophy of the entire gland, the increase in the weight of the tissue as we have already shown amounted to 65 per cent. This is due to a 40 per cent increase in the volume of the



TEXT-FIG. 2. Camera lucida outlines of central sections of adrenal medulla and cortex. (a) Normal adrenals of control groups. (b) Hypertrophied adrenals of uremic groups.

cortex. The increase in the weight of the whole gland in the female group (Table II) was 47 per cent and the increase in cortex volume 61 per cent. In Table VI these increases have been compared with the increases in weight and the chemical findings.

### *Histology.*

The adrenal glands from the uremic rats have been shown to contain a higher percentage of material soluble in fat solvents than those from the controls. This is in disagreement with the observations of Pfeiffer (6) who found that the amount of fat demonstrable microscopically in the adrenals of guinea pigs and rabbits which were made uremic by double nephrectomy was much less than normally present. A number of adrenals from uremic and control rats were therefore examined in frozen section with a fat stain (Sudan III).

The pictures obtained (Fig. 1) were similar to those described by Pfeiffer and did not bear out our chemical findings. In both sets of glands the medulla as usual contained no fat while the zona glomerulosa of the cortex contained the most. Between these two zones there was a gradual variation in the amount present but throughout the fat stain was evident in a much higher concentration and more evenly distributed in any given zone in the normal adrenals. We must conclude consequently that although material soluble in fat solvents is present in higher concentration in uremic adrenal glands most of this is of such a nature that it does not take the ordinary fat stains. The differences in staining and solubility suggest (15) that the decrease in the uremic glands is in cholesterol while there is an increase in saturated fatty acids which do not take the fat stain Sudan III.

On microscopical examination of paraffin adrenal sections (stained with iron-hematoxylin and eosin) from uremic animals a number of changes from the normal picture were found present. As we have noted, the cortex is much larger (Text-fig. 2) in the uremic adrenals. In a number of cases there were areas of hyperemia in the cortex and in two glands small hemorrhages surrounded by an area of necrosis were found. Hyperemia, hemorrhage and necrosis of the adrenal cortex have been reported before (6, 8) as a sequence of experimental uremia in guinea pigs, rabbits and dogs. In our experiments it formed an almost negligible part of the histological picture.

The cortical cells (Fig. 2) of the uremic adrenals were greatly enlarged. Their columnar arrangement was obliterated. The swollen cells did not stain as deeply or with a granular protoplasm such as is present in the cells of the normal gland. Nuclei of all the cortical cells were swollen and larger than normal. They stained more lightly than usual and in the fixed section were surrounded by unstained areolæ. In the reticular zone of the cortex (Fig. 3) the same differences between the cells in the control and uremic adrenals were found. In addition this part of the uremic glands was vacuolated by many dilated capillaries lined with endothelium but containing no cells or other stainable material. Although potentially present in the normal gland they are not evident except under very high power.

The medulla (Fig. 4) of the uremic adrenals showed distended



capillaries similar to those present in the adjoining cortical area. It is possible that the higher degree of adrenal enlargement found with the wet weights than with the dried glands may have been due to the fluid congestion of these areas. As has been noted by others (7), there was also evidence of degeneration in the medullary cells. The nuclei were swollen and stained less intensely than before and there was (6-9) a decrease in the chromaffin staining substance. The cells of the medulla were swollen. It would be supposed that this cellular enlargement in addition to the distended sinuses would give larger adrenal medulla volumes in the uremic than in the control rats and such was the case in the two experiments (Tables IV and V) in which this was determined. The increase in the medulla volume was however so small that it had no statistical significance.

#### DISCUSSION.

There is little reason to believe that the changes which have been found in adrenal glands following the production of an experimental uremia are unique for this condition. They are probably the manifestation of an intoxication and follow uremia because of the abnormal retention of metabolites. The cortical hypertrophy which ensued is perhaps analogous to the marked hypertrophy of the adrenal cortex known (1, 2) to accompany chronic infections and intoxications in animals. A somewhat similar cortical enlargement follows foreign protein injections (2) in the production of antisera and we have recently found an increase in the volume of the adrenal cortex of the rat as a result of chronic intoxication with morphine or bacterial vaccine.

In severe infections and intoxications pathological changes in the adrenal glands (6, 10, 11) occur regularly, varying in intensity from hyperemia, congestion and edema to hemorrhages and foci of necrosis. Changes of a similar nature in the uremic adrenals have been described. A decrease in the chromaphil staining of the adrenal medulla accompanied the uremia. This exhaustion is common to various intoxications and infections (6, 12).

The characteristic stainable lipids in the adrenal cortex show the same striking alteration in distribution (irregular) and amount (less) in uremia as in other intoxications (6, 12, 13, 14).

## SUMMARY.

The production of an experimental uremia in the albino rat by removal of both kidneys is followed by hypertrophy of the adrenal glands. In the case of male rats 90 days of age this adrenal enlargement amounted to 65 per cent and in the case of female rats 180 days of age 47 per cent.

The increase in the size of the whole gland is due entirely to hypertrophy of the cortex. This increase in the volume of cortical tissue amounted to approximately 40 per cent for males and 61 per cent for females and was due in large part to an increase in the size of the cells.

The content of water and material soluble in fat solvents was higher in the uremic than the control glands. However after subtraction of such storage materials a true hypertrophy of the cortex still remained. It amounted to 21 per cent.

Histologically the stainable fat had a more irregular distribution and was present in lesser amount in the adrenals from the uremic animals. The capillaries of the medulla and reticular cortex were distended. The nuclei of both the cortical and medullary cells were swollen and stained faintly.

## BIBLIOGRAPHY.

1. Langlois, P., Recherches sur l'altération fonctionnelle des capsules surrénales, *Arch. physiol. norm. et path.*, 1897, ix, series 5, 152.
2. Porak, R., L'hypertrophie et la teneur en adrénaline des surrénales dans les infections, les intoxications et certains états d'immunité, *J. physiol. et path. gén.*, 1919, xviii, 95.
3. Donaldson, H. H., The rat, Memoirs of the Wistar Institute, Philadelphia, 2nd edition, 1924, 255.
4. Landau, M., and McNee, J. W., Zur Physiologie der Cholesterinstoffwechsels, *Beitr. path. Anat. u. allg. Path.*, 1914, lviii, 667.
5. MacKay, E. M., and MacKay, L. L., Compensatory hypertrophy of the adrenal cortex, *J. Exp. Med.*, 1926, xliii, 395.
6. Pfeiffer, H., Über Veränderungen der Nebennierenorgane nach nervösen und toxischen Schädigungen, *Z. ges. exp. Med.*, 1920, x, 1.
7. Nakahara, T., Über Veränderungen des Nebennierenmarks nach Nephro- und Nephrektomien, *Virchows Arch. path. Anat.*, 1909, cxvii, 68.
8. Nowicki, W., Untersuchungen über die chromaffine Substanz der Nebennieren von Tieren und Menschen bei Nierenkrankheiten, *Virchows Arch. path. Anat.*, 1910, ccii, 189.

9. Borberg, N. C., Das chromaffine Gewebe. Nebennierenuntersuchungen II, *Skand. Arch. Physiol.*, 1913, xxviii, 91.
10. Oppenheim, R., Les capsules surrénales; leur fonction antitoxique, Thèse de Paris, Paris, 1902.
11. Goldzieher, M., Beiträge zur Pathologie der Nebennieren, *Wien. klin. Woch.*, 1910, xxiii, 809.
12. Loeschke, Untersuchungen über das Verhalten der Nebennieren bei Infektionskrankheiten, *Münch. med. Woch.*, 1910, lvii, 48.
13. Weltmann, O., Über das doppeltbrechende Lipoid der Nebenniere, *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 278.
14. Elliott, T. R., Pathological changes in the adrenal glands, *Quart. J. Med.*, 1914-15, viii, 47.
15. Kaufmann, C., and Lehmann, E., Kritische Untersuchungen über die Spezifitätsbreite histochemischer Fettdifferenzierungsmethoden, *Centr. allg. Path. u. path. Anat.*, 1926, xxvii, 145.

#### EXPLANATION OF PLATES.

##### PLATE 22.

FIG. 1. Adrenal sections from (a) control rat, and (b) uremic rat. Cut, stained and photographed under identical conditions. 10 micron frozen sections stained with Sudan III.  $\times 90$ . Shows the irregular distribution and diminished concentration of stainable lipids in the uremic adrenal cortex.

FIG. 2. Zona fasciculata of adrenal cortex, (a) control; (b) uremic. 6 micron paraffin sections stained with iron-hematoxylin and eosin.  $\times 350$ . Shows the swollen nuclei and cells in the uremic gland.

##### PLATE 23.

FIG. 3. Zona reticularis of adrenal cortex, (a) control; (b) uremic. 6 micron paraffin sections stained with hematoxylin and eosin.  $\times 350$ . Shows distended capillaries.

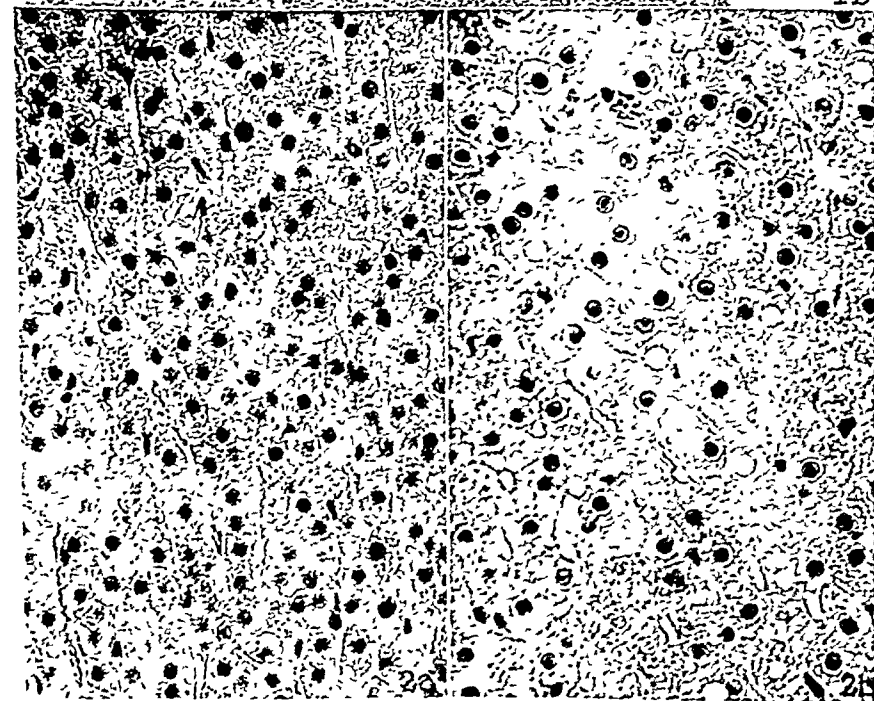
FIG. 4. Adrenal medulla. (a) control; (b) uremic. 6 micron paraffin sections stained with hematoxylin and eosin after chrome fixation.  $\times 350$ . Shows distended capillaries and large nuclei. The swollen cells contain a decreased amount of chromaffin substance.



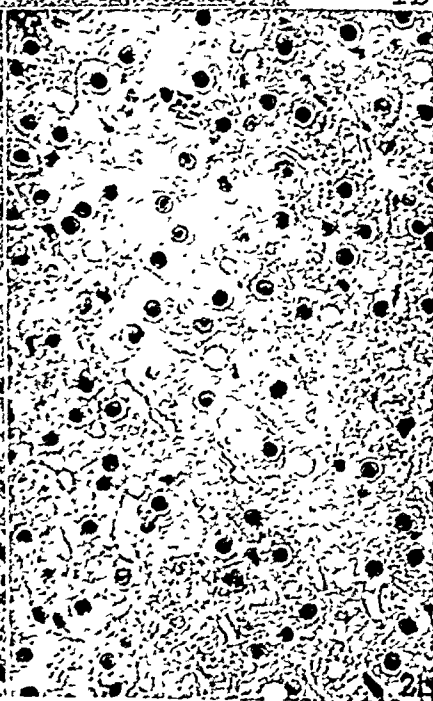
1a



1b

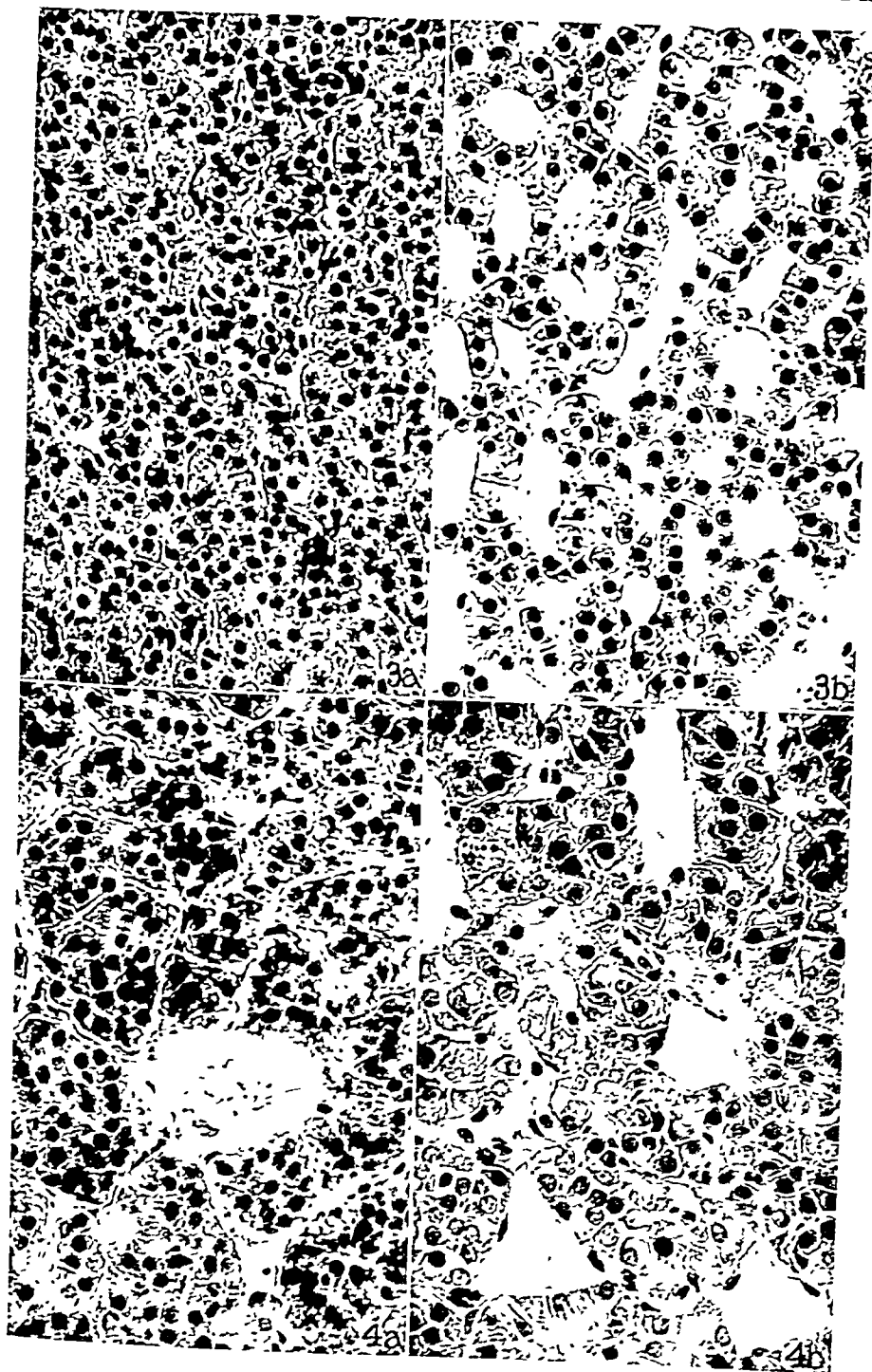


2a



2b





(MacKay and MacKay. Enlargement of adrenal cortex in uremia.)



# THE SYSTOLIC BLOOD PRESSURE OF THE NORMAL RABBIT MEASURED BY A SLIGHTLY MODIFIED VAN LEERSUM METHOD.

## FINAL REPORT.

By R. DOMINGUEZ, M.D.

*(From the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve University, Cleveland.)*

(Received for publication, May 9, 1927.)

In this report it is intended to complete and extend my previous publication (1) on the systolic blood pressure of the rabbit. An account of the method used, together with the technique both of the loop operation and of the measurements, is to be found in the first report and will not be given here.

The number of loops made has been 186, the first one on April 4, 1923, the last one on July 30, 1926. Good loops, that is, even, soft and of convenient length, were obtained in 97 (52.2 per cent), of which 4 were lost before any measurement was made (trauma, pneumonia) and 3 are omitted now because the measurements were started in the course of an experiment. The majority of failures is due to necrosis or infection of the loop. From March 31 to April 16, 1925, 23 loops were made, of which only 3 were satisfactory (13 per cent), 18 being spoiled by necrosis or infection (78.3 per cent). On the other hand, from September 25 to September 30 of the same year, 22 loops were made, of which 16 were good (72.7 per cent), only 5 being ruined by infection or necrosis (22.8 per cent). On July 29 and 30, 1926, 7 loops were made, all good. These figures show the extremes of failure and success which have been met in this work. The success of the other series falls between 50 and 60 per cent, so that the poor result with the loops made in April, 1925, seems to have been due to some extraneous factor not determined. The other causes of failure are general, such as pneumonia, pleurisy, otitis media, etc., and are not peculiar to this plastic operation. There is still another complication which may



TABLE I.

No. of rabbit	Sex	Observation period	No. of days on which blood pressure was taken	Mean of blood pressure 1st day	Maximum blood pressure whole period	Minimum blood pressure whole period	Pulse rate 1st day	Fastest pulse rate whole period	Slowest pulse rate whole period	Body weight		Remarks
										Initial	Final	
		1923		mm. Hg	mm. Hg	mm. Hg				kg.	kg.	
37-2	F	May 15-Sept. 12	92	126	165	102		272	172	2.110		Weight Apr. 4. Snuffles
39-6	F	May 15-June 1	11	125	136	90						Snuffles. Ear canker
39-7	F	May 16-June 5	15	130	170	125						Snuffles
42-0	F	July 25-Oct. 17	48	118	147	90	228	264	176		2.275	Albino. Litter of 10 Aug. 1
42-1	M	Aug. 3-Sept. 10	31	103.6	132	72	204	224	120	2.675		Weight June 5
46-1	M	Aug. 4-Oct. 17	40	104.1	130		208	256	160	2.390	2.600	Constriction. Snuffles
46-9	M	Oct. 16-Dec. 3	35	136.6	157	105	232	264	160	2.350	2.390	White Angora
47-0	M	Aug. 4-Jan. 16	103	102.4	149	90	194	248	128	2.375	2.805	
47-3	M	Aug. 18-Aug. 20	2	129.5	143	119	204	264	200	1.702		Weight July 24. Trauma
47-4	M	Oct.-Dec. 1	37	105.9	138	82	248	264	112	1.895	1.905	
47-7	M	Oct. 18-Nov. 26	31	101	133		162	236	136	2.120		Weight Oct. 27. Constriction. Abscess at angle of left lower jaw
48-2	M	Oct. 9-Dec. 1	44	111.9	129	75	242	264	156		2.060	
48-3	M	Oct. 8-Oct. 13	266	109.5	144	77	222	280	112	2.290	3.145	Sciatic injury, Oct. 18, 1923
48-4	M	Aug. 21-Oct. 12	16	114.1	127		240	300	208			Resistance. Abscess (flank). Constriction

		Oct. 18-Dec. 1	35	139.5	156	92	253	296	160		2.120	Scientific injury
		Aug. 15-Dec. 1	58	128.1	172	103	296	300+	192	2.285	2.610	Albino. Mediastinal abscess during experiment
		Aug. 14-Sept. 8	23	110	143	94	156	240	148		2.585	Abscesses in abdominal wall
		1923-24									3.015	
		Aug. 14-Oct. 13	301	134.5	201	116	248	300+	144	2.890		
		1924										
		Jan. 7-Feb. 26	44	99.2	135		200	220	136	2.310	2.855	Constriction
		Jan. 7-Feb. 11	31	126.8	140	95	200	280	132	2.290	2.000	Purulent pleurisy
		Jan. 7-Feb. 26	45	128.1	140		176	232	152	2.065	2.495	Constriction, marked
		Jan. 7-Mar. 27	39	144.8	160	103	192	300+	176	2.795	2.610	
		Jan. 7-Feb. 26	44	140.4	150		264	280	188	2.465	2.705	Constriction
		Jan. 7-Feb. 26	44	129.3	132		224	224	152	1.970	2.435	Albino
		Feb. 1-Mar. 27	46	138.7	157	95	192	240	136	2.345	2.275	
		Apr. 15-Apr. 29	10	136	159	124	240	256	216	2.275	2.305	
		Apr. 15-Apr. 29	10	143.6	162	125	232	256	184	2.060	2.050	
		Apr. 15-Apr. 29	9	154.6	162	135	216	216	176	2.175	2.200	
		Apr. 15-June 17	19	126.7	168	111	192	232	176	2.280	2.480	
		Apr. 15-May 15.	12	115.3	133		184	216	144	2.080	2.150	Constriction
		May 1-May 15	10	121	129		208	232	168	1.845	1.915	Constriction
		May 20-July 17	28	84.6	110	72	216	248	160	1.840	2.085	Resistance
		May 1-May 28	21	128.2	148		200	220	168	1.975	2.225	Constriction
		Apr. 22-May 20	20	122.9	135		216	240	184	1.815	2.000	Constriction
		June 13-July 11	23	109.9	131	91	168	224	144	2.055	2.310	
		Apr. 22-May 20	18	108	119		176	224	160	1.920	1.975	Constriction
		Apr. 23-Apr. 28	5	109.6	120	87	200	224	168	1.885	1.865	Trauma
		May 30-June 17	16	125.6	133	97	184	200	136	2.370	2.635	
		June 12-July 10	23	137.1	145	96	192	232	164	2.325	2.290	
		June 12-July 10	22	108	120	90	224	240	192	1.820	1.965	
		June 5-July 11	29	107	122	89	208	240	152	2.125	2.060	

## SYSTOLIC BLOOD PRESSURE OF NORMAL RABBIT

TABLE I—Continued.

No. of rabbit	Sex	Observation period	No. of days on which blood pressure was taken	Mean of blood pressure 1st day	Maximum blood pressure whole period	Minimum blood pressure whole period	Pulse rate 1st day	Fastest pulse rate whole period	Slowest pulse rate whole period	Body weight		Remarks
										Initial	Final	
				mm. Hg	mm. Hg	mm. Hg				kg.	kg.	
D 46	M	1924 June 12–July 11	23	135.1	149	105	184	232	160	2.184	2.115	Litter of 3 July 6 Ear cancer
D 49	F	June 30–July 19	16	116.7	140	97	184	232	168	2.140	2.365	
D 53	F	June 30–July 19	17	95.6	116	89	208	252	176	1.970	2.050	
D 55	F	June 30–July 19	17	123.1	155	107	216	248	168	2.425	2.215	
D 56	M	June 30–Aug. 22	43	101.7	132	84	208	248	160	1.955	2.165	Sciatic injury Litter of 8 July 16 Large abscess in thigh
D 59	F	June 30–July 19	17	115.8	145	98	196	232	176	1.910	1.965	
D 61	M	Aug. 11–Sept. 30	15	95.8	131	90	224	256	208	2.095	1.705	
D 62	F	July 8–Sept. 10	28	129.6	154	102	216	256	168	2.415	2.315	
D 65	M	Aug. 12–Sept. 10	25	138.2	170	112	192	240	168	2.150	2.325	Resistance, Constriction Otitis media. Constriction
D 66	M	Aug. 11–Oct. 27	61	100	125	70	208	280	144	1.945	2.460	
D 67		Aug. 1–Sept. 10	25	97.6	129	92	248	264	176	2.420	2.635	
		1924–25										
D 69	F	Sept. 25–Feb. 10	76	145	174	126	224	248	160	2.825	3.460	Resistance, Constriction Otitis media. Constriction
D 77		Nov. 10–Feb. 5	40	120.4	140	98	200	288	152	2.815	3.580	
D 78	M	Nov. 10–Feb. 5	39	114.5	156	91	264	280	168	3.115	3.730	
D 80	M	Nov. 10–Mar. 24	69	144.4	168	90	216	240	152	2.965	3.040	
D 82	F	Nov. 10–Feb. 10	43	110	135	90	224	248	168	2.325	3.355	Chewed loop
D 83	F	Nov. 10–Nov. 29	6	113.1	130	110	248	256	144	2.725	2.875	

D 84	F	1924-25	Nov. 24-Feb. 10	35	121.1	152	106	248	296	216	2.780	3.320	Weight Oct. 24, 1924, 2.270 kg.
D 85	M		Nov. 24-Feb. 10	33	103.7	141	99	200	248	144	2.820	3.350	
D 87	M	1925	Jan. 8-Mar. 24	59	110.8	130	82	264	264	148		3.625	
D 88		1924	Nov. 24-Nov. 26	3	109.6	115	94	200	200	176	2.480		Death from diarrhea
D 89		1925	Jan. 8-Mar. 26	60	118	142	97			160		3.195	Weight 2.300 kg. Nov. 22, 1924
D 91	F	1924-25	Nov. 24-Feb. 14	38	128.5	142		216	224	168	2.220	2.945	Constriction
D 92	F	1924	Nov. 24-Nov. 25	2	121.1	125	102	288	300	288	2.320		Accidental death
D 94	F	1924-25	Nov. 24-Feb. 10	34	126.3	167	112	232	288	200	2.310	2.460	Constriction
D 96	M		Nov. 24-Mar. 24	62	114.5	152		240	272	184	2.150	2.880	
D 97	F	1925	Apr. 15	1	133.5	140	130	248			3.480		
D 98	F		Apr. 15	1	131.1	138	124	272			2.550		Constriction
D 116	F		Sept. 24-Oct. 30	17	124.4	133	107	224	256	216		3.010	
D 118	F		Sept. 24-Oct. 30	16	123.9	138	95	232	256	208		2.330	
D 119	F		Sept. 24-Oct. 30	16	100.6	122	92	264	288	224		2.670	Weight Sept. 25, 1925. Trauma Constriction (late appearance)
D 123	F		Oct. 19	1	84.3	90	80	240			2.250		
D 125	M		Oct. 19-Oct. 30	10	101.7	119	89	232	240	216		2.320	
D 127	M		Oct. 19-Oct. 30	10	122.9	134	85	232	248	200		2.160	Trauma. Weight Sept. 29, 1925
D 131	M		Oct. 19-Oct. 30	10	97.4	112	72	232	240	184		2.080	
D 134	M		Oct. 20	1	71.3	75	69	208			1.720		
D 135	F		Oct. 19-Oct. 30	10	73.4	89	68	184	232	184		2.590	
D 136	M		Oct. 19-Oct. 30	10	82	105	80	176	208	176		2.510	
D 137	F	1926	June 10-June 22	9	114.2	129	102	192	208	176		2.920	

TABLE I—Concluded.

No. of rabbit	Sex	Observation period	No. of days on which blood pressure was taken	Mean of blood pressure 1st day	Maximum blood pressure whole period	Minimum blood pressure whole period	Pulse rate 1st day	Fastest pulse rate whole period	Slowest pulse rate whole period	Body weight		Remarks
										Initial	Final	
				mm. Hg	mm. Hg	mm. Hg				kg.	kg.	
D 138	F	1925-26 Oct. 19-Apr. 22	103	91.9	112	77	216	280	192	2.475	2.650	Constriction
D 139	F	1926 June 10-June 22	9	102.2	129	98	216	272	200		3.140	
D 140	M	1925-26 Oct. 19-June 22	111	101.7	132	74	200	264	184	2.065	3.410	
D 141	M	1926 Oct. 19-Apr. 22	92	91	126	79	208	280	184	1.885	3.060	Sciatic injury, Nov. 21, 1925
D 142	M	June 10-June 23	10	132.9	136	105	224	272	224		2.980	
D 143	F	1925-26 Oct. 19-June 22	107	106.3	138	85	240	288	216	2.310	3.470	
D 144	F	1926 Aug. 24-Oct. 4	10	100.7	115	90		240	208		2.565	
D 145	M	Aug. 24	1	90.4	92	89						
D 146	M	Aug. 24-Oct. 4	10	91.9	103	84	208	224	184		2.365	Trauma
D 148	F	Aug. 24-Oct. 4	10	77.4	98	70	208	240	200		2.835	Accidental death

appear when the animal kicks within the controlling box, and to which young animals, very quick in their actions and with soft bones, are specially susceptible. This is referred to below as "spinal trauma" and in Table I as "trauma." In some instances, under the same circumstances, instead of a complete paraplegia, there appears a partial

TABLE II.

Blood pressure	No. animals	Per cent
<i>mm. Hg</i>		
150-	1	1.1
140-149	6	6.7
130-139	13	14.4
120-129	20	22.2
110-119	18	20.0
100-109	16	17.8
90-99	10	11.1
80-89	3	3.3
70-79	3	3.3
	90	99.9

TABLE III.

Pulse rate per min.	No. animals	Per cent
280-299	2	2.3
260-279	5	5.9
240-259	13	15.3
220-239	17	20.0
200-219	29	34.1
180-199	13	15.3
160-179	5	5.9
140-159	1	1.2
	85	100.0

paralysis of one or both of the hind legs, without disturbance of the sphincters, and the animal recovers completely. This is referred to as "nerve injury." This complication is caused by the use of a controlling box and is not inherent in the loop method. One complication peculiar to the method is that some animals chew the loop. Five

did this: four (D 65, D 80, D 83 and D 96) during an interval in which no measurements were taken; and one (D 142) during a period of daily readings (March, 1927). The last one has not done it again so far, and three of the other four did not persist, so that in these animals the loop healed again and remained in good condition. But D 83 kept on chewing it until it became so scarred and so hard, that no more measurements could be taken. Eventually the animal died of hemorrhage. The reason for this is not clear, because repeated examination shows that there is no anesthesia or analgesia in the loop. Finally, one animal (D 25), on being lifted from the cage, caught its claw under the loop, kicked and tore it. This last accident (1 out of 97 good loops) and the "spinal trauma" are avoidable. The latter occurs in general on the first measurement and only in young animals, so it can be avoided by using full grown animals. This is preferable to packing the animal so tightly in the box that it cannot move. These are the only complications I have seen, which can be directly or indirectly attributed to the loop method. I have never seen thrombosis of the carotid or inflammation of the loop in animals under observation, which Van Leersum (2) reported having seen in one animal, or in fact any ill effects on the general condition of the rabbit. I have measured the blood pressure for as long as 15 months, with almost daily readings (1), and I am still measuring one rabbit 18 months after the first reading.

The data to be discussed now have been arranged in tabular form. They represent routine observations only, and not the results of any experimental condition imposed upon the animal, excepting of course the actual procedure of measuring the blood pressure and also a period of inanition of a few days to which several animals were subjected early in the work. Only one animal (No. 48-9) showed any effects of inanition, and the variations observed were not the extremes of the total variation recorded (1). On the other hand several of the animals developed diseases of various kinds, or were found to be pregnant or were the victims of some accident. These appear in Table I under "Remarks" and will be discussed later on. The blood pressure of a total of 90 rabbits (including the first 63 reported before) on the 1st day of observation is seen in Table II. The figures represent the arithmetic mean of the first 10 consecutive readings. The difference

between this table and the first one published (1) is partly due to better statistical treatment (a more careful distribution of the means). Three rabbits appear between 70 and 80 mm. Hg and none beyond 150 mm. The table, as it stands now, shows that on the 1st day of the examination, 60 per cent of the rabbits had a systolic blood pressure between 100 and 129 mm. Hg, and 85 per cent between 90 and 139 mm. Hg. The table, small as it is, compares in a general way with similar compilations made on men and women on the 1st day of examination. Concerning the published data on the blood pressure of the rabbit (direct measurement) it will be enough to quote the following: Volkmann (3) gives 90 and 108 mm. Hg. Meyer (4), after compiling the literature up to 1881, 51 experiments, comes to the following conclusion: "Dannach kann man als Grenzwerthe des mittleren Blutdrucks normalen Kaninchen ca. 70 und 140 mm. Hg annehmen." A remarkable conclusion, I think. Tigerstedt (5) says: "Beim Hunde schätzt man den Blutdruck auf 130-180 mm. Hg, beim Kaninchen auf 80-120."

The pulse rate of the rabbits reported in the present paper, counted on the loop itself at the end of each set of blood pressure readings, is shown in Table III. The pulse rate of the 1st day only has been given. By counting every other beat in a quarter of a minute, and multiplying by 8, it was possible to obtain the fast rates recorded. The error in these figures is at most  $\pm 8$ , small for the purpose. It appears that the pulse rate of 84.7 per cent of these rabbits ranged, during the first measurement, between 180 and 259 beats per minute. Tigerstedt (5) quotes the figures of Colin (1888), 120-150 per minute, and Ellinger (1894), 120-160 per minute, as the pulse rate of the rabbit. I have had no access to the original works quoted by Tigerstedt, so I do not know under what conditions Colin and Ellinger counted the pulse rate. But since it is much simpler to count the pulse than to measure the blood pressure, and since it can be done with very little disturbance to the animal, the pulse rate becomes an invaluable index of the presence of psychic or other factors which may have at the same time some effect on the blood pressure.

It is important to note that neither the mean of blood pressure nor the pulse rate of the *first* observation is in general the highest recorded during the whole period. Thus, for instance, only five



showed on the 1st day as fast a pulse rate as the fastest during the entire interval of observation of the respective animals (D 12, D 21, D 87, D 88, 48-7), and of these only one was near the maximum of the whole series (No. 48-7, 296 per minute), discarding of course those animals in which only one observation was made. With regard to the blood pressure, the number of animals in which the highest single reading of the corresponding period of observation was at most 10 mm. Hg higher than the mean of the 1st day, was sixteen, and of these eight were examined during less than 1 week. The remaining eight are D 10, 12, 21, 24, 34, 41, 116, 142. The distribution of the

TABLE IV.

Blood pressure	No. animals	Per cent
<i>mm. Hg</i>		
200-	1	1.2
170-179	4	4.9
160-169	7	8.5
150-159	10	12.2
140-149	15	18.3
130-139	22	26.8
120-129	12	14.6
110-119	7	8.5
100-109	2	2.5
90-99	0	
80-89	2	2.5
	82	100.0

maximum blood pressure by animals is seen in Table IV. By maximum is meant the highest blood pressure recorded *at least once* during the whole period of observation of the corresponding animal. Eight animals have been omitted because they were examined only during 1, 2 or 3 days. The table shows that on prolonging the observation period, 71.9 per cent reached 130 mm. Hg, 45.1 per cent reached 140, and 26.8 per cent reached 150, at least once, whereas on the first examination only 22.2 per cent were at or above 130 mm. Hg (see Table II).

I shall consider in more detail those which reached or passed 160 mm. Hg. D 8, D 19, D 21, D 65 and D 80 had only isolated high

figures, so that in their protocols there is no *mean* of 10 readings at or above 160. Nos. 37-2, 48-7 and D 94 had only one *mean*, and Nos. 39-7 and D 22 two *means* above 160 mm. Hg. D 69 showed *means* between 160 and 173, 11 times out of 76 (14 per cent), *scattered* throughout the period of observation. There remains one rabbit, No. 48-9, whose graph was reproduced in the first report (1). The number of *means* at or above 160 is 92, out of 516 (18 per cent), but what makes this observation noteworthy is the fact that, excepting scattered high means in October and December, 1923, practically

TABLE V.

Blood pressure	No. daily averages		Per cent	
	No. 48-3	No. 48-9	No. 48-3	No. 48-9
<i>mm. Hg</i>				
190-199		1		0.3
180-189		3		1.0
170-179		15		5.0
160-169		33		11.0
150-159		76		25.2
140-149		84		27.9
130-139	6	57	2.2	18.9
120-129	12	31	4.5	10.3
110-119	34	1	12.7	0.3
100-109	117		43.8	
90-99	79		29.6	
80-89	19		7.1	
Total.....	267	301		

all the readings made in September and the first third of October, 1924, were at or above 160 mm. Hg.

What is the behavior of the pulse rate? In general, when the blood pressure rises in a normal rabbit, the pulse rate increases, but the converse is not true: an increase in pulse rate is not necessarily accompanied by a rise in blood pressure. If the animal resists, whether the blood pressure rises or not, the pulse rate increases. In Table I the exceptional animals from the point of view of resistance are referred to by the word "resistance" under "Remarks." Under conditions of "excitement" there may be no effect on pulse rate or

blood pressure, or a marked increase in both or only in the pulse rate. *Excitement* is an unsatisfactory word to use, because it involves so much which is subjective. Without attempting to define it, I shall give a list of the observations included under this heading: tickling the animal's nose or closing its nostrils for a few seconds during a set of measurements; after a few days of inanition putting a piece of carrot under its nose, while measurements are being taken; placing adult male rabbits, after several months confinement, with females, both in heat and not in heat, and taking the blood pressure and pulse rate before and after copulation. Under these conditions "excite-

TABLE VI.

Pulse rate	Daily averages		Per cent	
	No. 48-3	No. 48-9	No. 48-3	No. 48-9
280-299		2		0.7
260-279	1	12	0.3	4.0
240-259		59	0.0	19.7
220-239	8	77	3.0	25.8
200-219	25	94	9.5	31.4
180-199	42	41	16.0	13.7
160-179	110	12	41.7	4.0
140-159	57	2	21.6	0.7
120-139	20		7.6	
100-119	1		0.3	
Total.....	264	299		

ment" is expected to be produced, and the animals show, indeed, signs which can be interpreted as such. Yet the results on blood pressure or pulse rate are not consistent or uniform in all the rabbits examined. A few examples may be seen in the first paper (1). The meaning of "an increase in pulse rate" will be understood from an examination of the figures of the two animals which exhibited the highest blood pressure, D 69 and No. 48-9.

D 69, as stated above, showed 11 means between 160.2 and 173 mm. Hg (with a mean of the means of 165) and the pulse rate of the corresponding days was between 208 and 248 per minute (mean 229). No. 48-9 showed 29 consecutive means between 155 and 193 mm.

Hg, with a mean of the means of 174, and the pulse rate of the corresponding days varied between 212 and 272 (mean 245).

The totals of the data from Nos. 48-9 and 48-3 are seen in Tables V and VI. Their graphs may be seen in the first paper (1). They represent the limiting cases found in this investigation. A few animals had a lower blood pressure than No. 48-3, but they were examined for a much shorter time, or their pulse rate was higher, or their figures were more scattered in distribution, or their behavior was not quite so good. Again, no other animal had a higher blood pressure than No. 48-9. So that these observations help in determining what might be called the boundary conditions of experimental hypertension in rabbits.

It is not practicable to present all the data in detail. If ever a compilation on the "normal" rabbit were attempted (and such a compilation is an urgent need of laboratory workers),<sup>1</sup> then this material would find its proper place there. It is only necessary here to show the salient facts observed on which a criterion for a pathological rise in blood pressure in the rabbit may rest. It must be borne in mind, that by "normal blood pressure of the rabbit" is meant the systolic blood pressure of a healthy looking rabbit *under the conditions of the measurement*. The practice of taking the blood pressure of the rabbit a few days before the experiment, *as control*, has less value than is generally conceded. 1 week, or 1 month of observation, is not sufficient to characterize completely the blood pressure curve of a rabbit, and does not guarantee that the pressure will behave in the same way in a subsequent interval of time. Finally, the purpose of the investigation must be considered. For acute experiments, there is little need of a carotid loop. On the other hand, a method like this is indicated for the study of prolonged alterations in blood pressure, which may simulate either the clinical picture of hypertension or Addison's syndrome. With these reservations in mind, a criterion could be formulated as follows:

(a) Under the conditions of the measurement, a rabbit may be said to have a pathologically high blood pressure, if the blood pressure

<sup>1</sup> The recent work of Brown, Pearce and Van Allen is an excellent beginning in this direction (11).

oscillates *above 180 mm. Hg* and does not fall below that figure during a length of time dependent on the nature of the experiment.

(b) Under the same conditions, the blood pressure may be considered pathologically high if it oscillates *about 170 mm. Hg* with a concomitant pulse rate *below 200 beats per minute*.

The introduction of the pulse rate in (b) allows the avoidance of consideration of resistance, "excitement," and the like, and makes unnecessary the discarding of any figure in the course of the investigation. It seems superfluous to add that these criteria are provisional. Strictly speaking they rest on observations made on 90 "normal" animals (over 30,000 blood pressure readings, about 2,900 pulse rate counts), but they are reinforced by the subsequent experience with these animals under several pathological conditions. Since these observations, moreover, spread over 4 years, and since the animals were obtained from different dealers in different years, it is expected that future experience with the method here adopted will not differ grossly from that described in the present report. The observations of Van Leersum himself (1911, 1912) on twelve rabbits, agree very well with mine.

*An Important Source of Error of the Method.*—The following phenomenon has been observed in several rabbits.

D 138, Feb. 4, 1926, 3.09 p.m.\* 100-101-79-82-84 = 93-98-98-100 = 103-97-100-99 = 102-101-103-103 = 103-92-92 (pulse rate 232).

D 80, Jan. 21, 1925, 10.55 a.m.\* 152-102-112-108-112-118-126 = 141-132-132-136 = 146-143-143-145 = 145-143-142 = 141-142 (pulse rate 224).

Jan. 30, 1925, 11.36 a.m.\* 144-140-140-138 = 139-139-140-105 = 102-108-116-120 = 130-131-130-135 = 130-132-128-132 (pulse rate 224).

\* Cuff adjusted to loop.

This abrupt fall in blood pressure occurs at any time in a given rabbit. If observed once in an animal it may be observed many times. No sign of a more general character accompanies this phenomenon: no alteration in pulse rate, no change in behavior, no respiratory disturbance, no pupillary effect. It may probably be explained by a local constriction of the carotid under the influence of

the pressure applied on the cuff. The fact that it does not appear in all animals is not an objection to this explanation, because the carotid is subjected to this stimulus intermittently for a short time (3 to 5 minutes), and it is not to be expected that all carotids will react in the same way to a given stimulus. It is clear that the change must be local and not the expression of an actual fall of arterial pressure, which would be accompanied by a change of pulse rate. In Table I it appears under "Remarks" as "constriction." In some animals it is very slight, for instance:

D 78, Nov. 17, 1924, 3.18 p.m.\*  $138-132-133-132 = 132-130-96 = 129-128-127-127$ .

In others it is frequent and becomes disturbing, as in the following example:  
D 6, Jan. 26, 1924, 10.27 a.m.\*  $115-110-104-89-88 = 112-113-115 = 114-112-$   
(pulse rate 184).  
 $114-112-113-97 = 106-112-106-108 = 112-116$   
(pulse rate 176).

\*Cuff adjusted to loop.

I have discarded no animal on this account, but the "minimum blood pressure" has no meaning under these conditions, and has been omitted from the table. It is enough to recognize this phenomenon to avoid the error produced by it.

*Changes in the Carotid within the Loop.*—The majority of the carotids enclosed in a loop showed after the death of the animal a marked transverse striation of the intima. The striations are due to a slight thickening of the intima. Under the microscope the lesion is not well defined: a slight sclerosis of the subintimal tissues and here and there a moderate vacuolization of the muscular coat. This striation has been observed in the remaining parts of several carotids, a long time after the loop had been severed on account of necrosis; that is, in arteries to which *no cuff had ever been applied*. It has been found absent in arteries in which measurements had been taken for a considerable time. In a few animals which died immediately after the operation (possibly ether death), the examination of the carotid revealed sharp transverse lines of intimal tearing, very probably produced by stretching the artery during dissection. Before this injury was recognized and avoided, it was not uncommon to see, before the final suture of the loop, small extravasations of blood on the muscular layer of the carotid, which gave to the artery a slightly beaded appear-

ance, the more noticeable since the artery at the end of the operation contracts down through exposure. With more care in the manipulation of the artery this lesion may be reduced in extent, and possibly eliminated altogether. The fact is, the rabbit's carotid is a very thin and delicate object and must be treated accordingly. I think that the lesion observed in a good number of the carotids is due to the cicatrization of these intimal operative injuries.

Shapiro and Seecof (7), reviewing some of the methods of blood pressure measurement in the rabbit, say that Van Leersum's would be an excellent one "provided the artery could be thus isolated without altering the compressibility of its walls through inflammatory reactions. This objection seems to render the method both impractical and prohibitive." The authors fail to realize that the method is not sensitive enough to detect small changes in the thickness of the artery. The cuff effect in this method consists of several parts, the cuff proper (a piece of cotton fabric and of rubber tubing), the skin with and without hair, subcutaneous tissue and the vessel wall. Of these, the first three become softer and more supple in time, which, theoretically at least, would lower the value of the total effect, whereas the thickening of the intima is not progressive as far as I have been able to ascertain. Under these conditions, fluctuations of 40 mm. Hg *in one animal* several days, or several weeks apart, or occasionally in 1 day, cannot be due to variation in compressibility of the arterial wall. Finally, in experiments on arteriosclerosis (to be reported soon) where *calcification* was found in the media of the carotid within the loop, as well as in other large vessels, and in the aorta, no constant elevation of the blood pressure was observed.

MacWilliam in his review (8) on the blood pressure of man says: "It may be taken as established that high blood pressure readings, when carefully taken, represent approximately correct measurements of the actual intra-arterial pressures as a rule. It is only in a small minority of abnormal cases of thickened arteries with excessive tonic contraction, etc., that serious discrepancy may occur, sclerotic conditions without muscular contraction having no important influence. Digital compression for 3 or 4 minutes or massage of the artery are useful in removing abnormal resistance and have the advantage of not causing congestion of the limb which may arise from repeated compressions by the armlet." The lesion in the carotid of my rabbits is of the type considered by MacWilliam as having no important influence, and the method, as actually used, amounts to a digital massage of the artery, so that, if this massage were as efficacious as MacWilliam believes and such "excessive tonic contraction" were ever present in the rabbit, the method would automatically eliminate this source of error.

*Blood Pressure in Pathological Conditions.*—After what has been said before it will be easy to see the difficulty of attaching importance

to small variations in blood pressure, within the range of figures between 80 and 170 mm. Hg. Their importance increases as the pressure approaches these limiting values. The diseases that have occurred in animals under observation (not experimented upon, excepting the blood pressure readings) are, in order of decreasing frequency: coryza, ear canker, pneumonia (with or without pleurisy), purulent pleurisy, subcutaneous abscesses, meningitis (drooping and rotation of head). Diseases found at autopsy, not diagnosed during life: coccidial cysts in abdomen, otitis media (one or both sides, without meningeal involvement), scarred kidneys, arteriosclerosis of aorta (slight), pulmonary abscesses (discrete), pulmonary mycosis, mediastinal abscess. Accidental injuries: "spinal trauma," "sciatic injury," total infarction of kidney (ligature of renal vein during adrenalectomy).

Of all these conditions only two deserve special mention: "Spinal trauma" and meningitis. During the terminal coma of the latter the blood pressure is low, 70-80 mm. Hg. Occasionally, however, the pulse is slow and irregular, and the actual blood pressure readings are not reliable. Concerning "trauma," the observation is as follows: the animal is within the box, quiet; blood pressure is taken as usual. Suddenly the animal moves within. The motion may not appear to be of excessive violence. The blood pressure rises abruptly (140-150 mm. Hg) and the pulse is very slow and strong. Soon afterwards (1 minute or so) the pressure gradually falls and the pulse rate increases. Greater details cannot be given because the occurrence fortunately is rare and takes place within a very short time.

In the other conditions the blood pressure findings are discordant. In one rabbit suffering from a large unnoticed abscess of the thigh, the blood pressure was low (the graph has been reproduced in another paper (9)). During the course of pleuropneumonias the blood pressure may lie between 80 and 90 (see graph for No. 47-0 (10)), or between 90 and 110 mm. Hg. In No. 48-7 the mediastinal abscess, a large mass twice as large as the heart, situated on the right side, anteriorly, and adherent to the pericardium, showed a certain effect on the blood pressure, the more interesting since it was completely misunderstood during life. The observation is so unusual, that an abstract of the protocol is presented.



No. 48-7, albino, male rabbit. July 31, 1923, carotid loop is made, weight 2.440 kilos. Partial necrosis of distal portion of loop. Healed completely.

Aug. 15, first blood pressure readings (see Table I). Aug. 18–Sept. 8, blood pressure between 120 and 129 mm. Hg (74 per cent of daily means). Oct. 6 and Dec. 2, 1923, blood pressure between 130 and 149 (72.5 per cent of daily means.)

Dec. 3, lead carbonate smeared in carrot, fed by hand, about 25 mg. daily. Dec. 8, weight 2.550 kilos; Dec. 23, best weight, 2.675 kilos. Dec. 24, daily dose of lead carbonate increased to 78 mg.

Jan. 4, 1924, diminished appetite; Jan. 12, weight 2.480 kilos; Jan. 17, weight 2.190 kilos. Jan. 20, little appetite. Jan. 26, lowering of blood pressure; blood pressure from Dec. 3, 1923, to Jan. 24, 1924, between 120 and 139 (78.4 per cent of daily means). Feb. 6, lowest weight, 2.120 kilos.

The loss in weight and the lowering of blood pressure (see below) were thought to be due to lead carbonate, and the latter was interrupted until Feb. 18, 1924. From Feb. 18 to May 31, lead was given daily in increasing dose, from 40 up to 300 mg. Feb. 16, weight 2.260 kilos. Weight increases slowly in spite of increasing dose of lead.

Feb. 20, symptoms while drinking water, as if water had entered the trachea. Abundant râles. Bronchial (or tracheal) moisture persisted until Mar. 19. On Mar. 1, it was thought that the animal had a foreign body in the trachea. Apr. 11, weight 2.480 kilos; May 26, weight 2.285 kilos. June 2, animal looks sick. Blood pressure from Jan. 26 to May 31, 1924, between 100 and 119 mm. Hg (87 per cent of daily means). Concerning the pulse rate the only thing at all remarkable is the fast rate in the second half of May, between 240 and 280 per minute (mean 258). Blood pressure on June 2, between 74 and 78 mm. Hg, pulse rate 208. June 3, lies on side, does not move. June 4, dead.

The beginning of this disease may have been concomitant with the first fall in weight in the latter part of Dec., 1923, and the formation of a mass in the mediastinum (probably from lymph nodes) may account for the spell on Feb. 20 and the slow recovery from it. The increase in weight in spite of increasing dose of lead carbonate militates strongly against lead poisoning. Lead given in this fashion to a group of animals (of which No. 48-7 is one) has shown no deleterious action (experiments to be reported later). The animal was in excellent physical condition up to June 2, 1924. It had been mated repeatedly during the whole observation, the last copulation having occurred on May 20.

*Carotid Loop in Dogs.*—As a further check on the method, loops were made in two dogs. Here it was possible to use the stethoscope and find auscultatory criteria for systolic and diastolic pressure similar to those used in man. The daily means of one of the dogs ranged from 123 to 165 mm. Hg, 77.7 per cent lying between 130 and 149 (85 days), for the systolic pressure, and from 69.1 to 103 mm. Hg, 87 per cent between 80 and 99, for the diastolic. The pulse rate oscil-

lated between about 60 and 184 per minute, 87 per cent of the counts being between 80 and 139. The other dog was examined during a much shorter time: systolic between 125.8 and 164.5 mm. Hg (10 daily means), diastolic 80–103, pulse rate 104–160. Both these dogs were subjected later on to double adrenalectomy. A complete report will appear shortly in collaboration with Dr. J. M. Rogoff.

#### SUMMARY.

The blood pressure and pulse rate of 90 normal rabbits have been studied for various periods of time, from 1 day (accidental death interrupting the observation) to 15 months. The main data are presented in a table containing the blood pressure and pulse rate on the 1st day of observation, the maximum and minimum of both during the entire period of observation of each animal, together with the sex and weight of the animal. Separate tables are given showing the distribution of blood pressure, pulse rate and the "maximum" blood pressure by animals. Detailed data on two animals observed for the longest time are given in tabular form. The anatomical changes that occur in some carotids enclosed in a loop are described and discussed. Considerations on "excitement" and pathological conditions which arise spontaneously in rabbits are given. A criterion for a pathologically high blood pressure in rabbits is proposed.

The blood pressure of the normal rabbit ranges between 70 and 170 mm. Hg. The pulse rate, taken simultaneously with the blood pressure, fluctuates between 112 and 300 per minute.

I acknowledge my sincere thanks to Dr. G. N. Stewart for his valuable criticism throughout the work.

#### BIBLIOGRAPHY.

1. Dominguez, R., *J. Metabol. Research*, 1924, vi, 123.
2. Van Leersum, E. C., *Arch. ges. Physiol.*, 1911, cxlii, 377.
3. Quoted by Tigerstedt (5).
4. Meyer, H., *Arch. exp. Path. u. Pharmacol.*, 1881, xiv, 313.
5. Tigerstedt, R., *Physiologie des Kreislaufes*, Berlin, 1922, iii, 154.
6. Van Leersum, E. C., *Z. exp. Path. u. Therap.*, 1912, xi, 408.
7. Shapiro, S., and Seecof, D. P., *J. Lab. and Clin. Med.*, 1925, x, 826.
8. MacWilliam, J. A., *Physiol. Rev.*, 1925, v, 303.
9. Rogoff, J. M., and Dominguez, R., *J. Metabol. Research*, 1924, vi, 141.
10. Dominguez, R., *J. Exp. Med.*, 1927, xlvi, 463.
11. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1926, xliii, 733.



## EXPERIMENTAL ATHEROSCLEROSIS AND BLOOD PRESSURE IN THE RABBIT.

By R. DOMINGUEZ, M.D.

*(From the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve University, Cleveland.)*

(Received for publication, May 9, 1927.)

The relation between atherosclerosis and blood pressure has engaged the attention of several workers following the statement of Fahr (1) and Van Leersum (2) that rabbits fed on abnormal diet (egg, liver) develop a high blood pressure. The published material that I have examined is disconcerting, due not so much to the variety of methods used to determine the blood pressure, as to the lack of sufficient information concerning the "normal" fluctuations of blood pressure, that is, the fluctuations observed in normal animals with the particular method used. Van Leersum, who claimed to have found a marked elevation of blood pressure under the influence of a liver diet, obtained no lesion whatever in the circulatory apparatus of his rabbits. Again most workers seem to be under the impression that the blood pressure is a constant, that is, that if the blood pressure oscillates in a certain region for a few weeks before the experiment, then any increase above this region that may occur afterwards during any experimental condition is necessarily due to the experimental condition, although these values may be well within values recorded from other normal animals. I have had considerable experience with Van Leersum's method (3, 4) and have given curves which sufficiently illustrate the fallacy of that assumption. For instance, in the graph of Rabbit 48-3 (3) it may be seen that the blood pressure oscillated around 100 mm. Hg for fully 10 months (from November, 1923, to September, 1924) and then rose in September and October, reaching 140 (average) on September 27. If an experiment had been started in the last week of August and this rise had been observed, the observation would have been supported by a good control period, but an inaccurate conclusion could have been drawn. Another type of curve is shown here, and

still others may be found in former papers (3-5). To avoid repetition, by "blood pressure" and "normal range" is understood the systolic blood pressure of the rabbit as obtained from a carotid loop (Van Leersum's method) and the range of blood pressure in normal rabbits as determined previously by the writer.

The experimental atherosclerosis of the rabbit has in itself considerable intrinsic interest, so it seemed worth while to repeat the experiment. The literature on cholesterol feeding experiments is voluminous. For an introduction to the subject the reader is referred to the references given here, particularly Schönheimer. In the present work egg yolk was chosen since natural emulsions have been found best suited for the purpose. The rabbits (five in number) were given their usual food (hay, oats, greens) throughout the experiment; the yolk of one or two eggs was mixed with powdered unleavened bread and dried at 37°C., the whole appearing finally as yellowish crisp masses. The animals ate it readily in the beginning, but after some time they seemed to tire of it, so the yolk was diluted with plain water and the stomach tube used. The blood pressure was taken daily in the manner explained elsewhere (3). These five animals were chosen at random. One had been measured as a routine for several months, others for less time. Two of the five received lead carbonate by mouth in addition to the egg yolk. It seems justifiable to report the two cases where lead was also given, D 1, D 10, for the following reasons:

1. The association of lead poisoning and high blood pressure in man has always been considered in clinical medicine.

2. Lead carbonate in the form given to these animals and lead acetate given by stomach tube, in my experience, do not produce high blood pressure in the rabbit (unpublished data).

3. Recent work done in this country on the general subject of lead poisoning throws doubt on the efficiency of absorption of lead by the gastrointestinal canal (summarized in Reference 6).

4. There is no essential fact in the behavior or in the autopsy of these two animals that could be attributed with certainty to lead.

The curves were plotted at the completion of the experiment. The organs of the animals were carefully examined after death. Microscopic examination, however, was not systematically done. A brief analysis of Van Leersum's report (2) will be found at the end of the

paper, followed by a note on the results obtained by other methods of measuring the blood pressure.

The essential data on these five animals will be given in the form of condensed protocols.

*D 1.*—Male, brown, Belgian rabbit. Nov. 30, 1923, carotid loop is made; weight 2.310 kilos. Jan. 7, 1924, blood pressure measurements started; weight 2.625 kilos.

Feb. 26, feeding experiment began; one egg yolk mixed with powdered "Matzos" and 30 mg. of lead carbonate smeared in carrot and fed by hand, daily; weight 2.855 kilos. Mar. 13, daily dose of lead carbonate increased to 60 mg. Mar. 29, daily dose of lead carbonate increased to 80 mg. Apr. 6, best weight 3.360 kilos.

Apr. 14, conjugate motions of eyes and head, toward the right, with drooping of right ear. Lead and egg feeding are interrupted. Apr. 16, same condition; weight 2.660 kilos. Apr. 21, right ear is full of a foul smelling creamy pus; weight 2.410 kilos. Apr. 30, eyes were found to possess well developed corneal arcs. May 11, weight 2.395 kilos. May 17, death in coma.

Total number of yolks consumed, 40. Total amount of lead carbonate given, 2.690 gm.

*Blood Pressure.*—Highest pressure recorded was 135 mm. Hg on February 7. This animal was one of those which exhibit a phenomenon described in the preceding paper (4) and ascribed to a local constriction of the carotid under the stimulation of the external pressure applied on the cuff. It is well illustrated by the following examples.

Feb. 6, 1924, 10.21 a.m.\* 121-71-101-112 = 115-126-125-128 = 125-125-120-123 = 128-124-122-120 = 121-122-124-127 (pulse rate 192).

Mar. 4, 1924, 10.09 a.m.\* 103-88-68-0 (15 seconds)-99-92-93-93 = 96-95-93-95 = 76-69-70-89 = 95-93-94-91 = 92-91-97-95 = 95-95-96-93 (pulse rate 168).

\* Cuff adjusted to loop.

This phenomenon renders the tabulation or plotting of the figures almost impossible. It appeared throughout the course of the experiment, but not every day. It was absent in the last part of the experiment, when the intracranial complication of the otitis media became evident. The blood pressure during this latter period was, in general, low, oscillating between 70 and 90. The pulse rate varied between

136 (February 23 and April 23) and 220 (January 17) per minute. During the terminal coma the pulse became very irregular, a few beats passing through at 90 mm. Hg.

*Autopsy.*—Heart: base of large mitral cusp infiltrated with fatty substances. Aorta: large patch of infiltration at opening of arch branches, extending for a short distance into the common root of carotids and into left subclavian. In ascending arch there are a few minute nodular elevations. Nothing in thoracic aorta. In abdominal aorta, several small nodules and two streaks, one at root of celiac trunk, another at level of renal arteries. These two streaks are perpendicular to the axis of the aorta. Remainder of aorta and iliacs, normal. Pulmonary artery shows several elongated patches of moderate size, along posterior wall, parallel to the axis. Left adrenal, 950 mg.; right 860 mg. (weighed on March 26, 1927, in formol in the interval, see discussion). Corneal arcs, bilateral, well formed. Brain: white, thick, purulent exudate at level of tentorium cerebelli, both sides of midline. Right middle ear is filled with a creamy pus.

*D 10.*—Female, brown rabbit. Dec. 6, 1923, left carotid loop is made; weight 2.280 kilos. Jan. 7, 1924, blood pressure readings started; weight 2.465 kilos.

Feb. 26, feeding experiment began; one egg yolk mixed with powdered "Matzos" and 30 mg. lead carbonate smeared in carrot and fed by hand, daily; weight 2.705 kilos. Mar. 7 and 12, best weight 2.840 kilos. Mar. 13, daily dose of lead carbonate increased to 60 mg. Mar. 26, animal looks sick; weak; egg and lead are withheld. Mar. 27, weight 2.555 kilos. Apr. 16, egg given again; animal has recovered its former appearance and behavior, but not its weight; weight 2.515 kilos. Apr. 26, weight 2.700 kilos.

May 1, egg given through stomach tube; lead carbonate given again, 50 mg. daily. May 2, no corneal arc in either eye. May 15–17, lead carbonate suspended in egg yolk emulsion, stomach tube. May 16, weight 2.350 kilos. May 18, dead.

Total number of egg yolks consumed, 55. Total amount of lead carbonate given, 1.780 gm.

*Blood Pressure.*—Highest figures recorded before experiment: 146 (January 7, 1924), 150 (January 23), 149 (February 25) with averages for day, 140.4, 140.3, 144.8 respectively. Highest figures recorded during experiment: 152 (March 5), 150 (March 6), 149 (March 11), 148 (March 12), 150 (March 15) with averages for day, 139.6 (30 readings), 143.2, 145.9, 140.6, 143.0 respectively. From March 26 to April 10, the blood pressure reached the lowest level observed during

the whole experiment, as low as 87 mm. Hg. This is the same period in which the animal appeared sick, concomitantly with loss in weight, loss in appetite, and, as it will be seen afterwards, increase in the pulse rate. I have no explanation for this. I have seen nothing like it in my experiments with egg yolk alone, or in the other animal which received lead together with the egg, or in several animals which have had lead carbonate or lead acetate alone (unpublished data). The pulse rate during this period oscillated between 216 and 288 per minute. The contrast in the behavior of pulse rate and blood pressure in the three periods, before March 26, from March 26 to April 10, and after April 10, is best seen in tabular form, where I have taken figures corresponding to the two extremes and mean of pulse rate for the respective periods.

Date	Pulse rate per min.	Blood pressure
<i>1924</i>		<i>mm. Hg</i>
Jan. 7	264*	138-146
Feb. 5	264	119-130
Feb. 9	272	118-124
Feb. 25	280	139-149
Jan. 9	232	116-138
Jan. 22	240	110-130
Mar. 17	232	130-135
Feb. 11	188	101-116
Mar. 26	216	87-92
Mar. 31	264	98-107
Apr. 5	280	97-101
Apr. 7	288	93-103
Apr. 16	192	102-110
May 1	144	111-126
May 14	240	122-129

\* These fast rates are counted by groups of two  $\frac{\downarrow \downarrow}{1}$   $\frac{\downarrow \downarrow}{2}$   $\frac{\downarrow \downarrow}{3}$ .

With increasing rates the pulse becomes very rhythmic and this process of computation is accordingly easier.

*Autopsy.*—Aorta is mottled throughout with yellow spots and streaks, slightly elevated, parallel to the axis of the vessel; somewhat more abundant in arch and thoracic portions. For a distance of 1.5



cm. above the opening of the celiac trunk these small infiltrated areas become confluent. Infiltration from this point downward is less and less marked. Pulmonary artery shows a large, irregular, slightly raised patch, at bifurcation, extending both ways for a short distance. In left kidney there are a few yellow streaks in outer zone of pyramid. Eyes show no visible corneal arcs. Adrenals are large, right weighs 440 mg. (weighed on March 26, 1927, almost 3 years in formaldehyde solution; left adrenal has been split open and a piece of central portion cut off for microscopic examination; there was no obvious difference in size at the time of the autopsy).

*D 6.*—Female, brown rabbit. Dec. 4, 1923, carotid loop is made; weight 1.785 kilos. Jan. 7, 1924, first blood pressure readings; weight 2.065 kilos.

Feb. 26, feeding experiment began; one egg yolk mixed with powdered "Matzos," daily; weight 2.495 kilos. Apr. 3rd and 4th weeks, rut. Apr. 26, best weight 2.820 kilos. Apr. 30, egg yolk given by stomach tube.

May 2, corneal arc is well formed in right eye, spreading toward center of cornea for a distance of 3 mm. from limbus. In left eye there begins to appear a faint, delicate, white line next to iridocorneal junction. June 28, weight 2.340 kilos.

July 14, two egg yolks by stomach tube, daily. Aug. 19, weight 2.165 kilos.

Aug. 21, animal is cold, wabbly, looks sick. Egg feeding is discontinued. Aug. 22, very weak, cold. Died at 11.30 p.m.

Total number of egg yolks consumed, 210 (without interruption, except isolated days).

*Blood Pressure.*—Range of figures where highest values were obtained, (a) before egg yolk feeding, (b) during egg yolk feeding, together with mean of set and pulse rate:

Date	Oscillation	Mean	No. readings	Pulse rate
1924	<i>mm. Hg</i>	<i>mm. Hg</i>		
(a) Jan. 14	128-134	130.4	10	168
Jan. 15	129-134	130.4	10	160
Feb. 26	128-140	133.7	10	
(b) Mar. 6	124-139	131.4	20	176
Mar. 27	105-135	115.2	20	160
Apr. 10	118-138	128.6	20	216
Apr. 29	122-143	136.1	19	216
May 3	114-142	134.2	20	216
May 6	120-141	130.9	20	200

Toward the end of May, during the whole of June and first half of July, the pressure reached the lowest values observed during the entire experiment, oscillating between 72 and 108 mm. Hg. Pressure above 120 was recorded on May 17, and the next pressure above 120 was recorded on July 18. The lowest temperature of the room where the measurements were done, was, in this interval of time, 19°C. (May 22 11.05 a.m.), blood pressure 86–109, mean 97.8 (20 readings), pulse rate 152 per minute. The highest temperature in the same interval 30°C. (June 24, 3.53 p.m.), blood pressure 85–93, mean 90.1 (10 readings), pulse rate 184 per minute. The thermometer is mounted on the stand of the manometer and the temperature read systematically at the end of the measurements. From July 18 on, the pressure was not as low as in the interval just discussed but reached levels in general not as high as those recorded before May 17, the only exception occurring on August 9, when the pressure rose to 141 after the rabbit moved during the measurement. The fastest pulse rate was 232 per minute, on February 19 and March 12, which did not coincide with the highest pressures. The lowest rate was 128 per minute, on June 28, with a blood pressure between 80 and 86, mean 83.1 (10 readings), and on August 22, when the rabbit was profoundly asthenic and the blood pressure was between 82 mm. and 90 mm. Hg (measured while animal was lying on its side).

*Autopsy.*—Marked dilatation of heart. Marked atherosclerosis of aorta at root, arch and first portion of thoracic aorta, where intima is thoroughly infiltrated; from here on infiltration is patchy, mainly at the opening of branches, with a rather large patch at the opening of celiac trunk. The infiltration is greatest just beyond the opening of branches, in many instances forming like a crescent on the caudal side of the opening. Lumbar aorta is practically free; iliacs free. Carotids not involved, except at their opening, and excepting a small portion of the root of left carotid (that within loop), in continuation with the aortic infiltration. Aortic leaflets and large mitral cusp are slightly infiltrated. Pulmonary shows a large patch at bifurcation, extending both ways for a short distance. Root of pulmonary is completely free. Profound infiltration of liver, which feels hard, and in many areas is coarse, the capsule in these places being thick and opaque. Spleen large and pale. Adrenals are large; weighed on March 26, 1927

(after almost 3 years in formaldehyde solution), left 550 mg., right 500 mg. Ovaries thoroughly infiltrated, but scarcely larger than normal. Gall bladder much distended, walls not thickened. Bile is very thick. Lungs contain many whitish nodules throughout, not unlike a miliary tuberculosis, and small areas of congestion at both bases. The microscope revealed the presence of a radiating fungus in these nodules.

No. 47-0.—Male, gray, Belgian rabbit. July 7, 1923, left carotid loop is made; weight 2 kilos. July 31, first blood pressure readings. Jan. 12, 1924, weight 2.895 kilos.

Jan. 16, feeding experiment started; one egg yolk mixed with powdered "Matzos," daily. Feb. 16, best weight 3.360 kilos.

Apr. 30, well developed corneal arcs on upper segment of both corneae. Egg yolk by stomach tube. May 2, corneal arcs spread to lower segments, equatorial parts remaining free. May 11, weight 2.645 kilos. May 12, looks sick. May 15, dead.

Total number of egg yolks consumed, 117 (without interruption).

Concerning the *blood pressure*, see Fig. 1 and discussion.

*Autopsy*.—Aorta thickened and opaque, intima greatly thickened, yellowish, rough and totally infiltrated from arch down to bifurcation; only spot free from atherosclerosis is the root of the aorta where there is only a small elevated nodule. Pulmonary artery is almost as much involved as the aorta. Root of carotids and iliacs infiltrated. Small amount of fluid in abdomen. Walls of descending and transverse colon are yellowish. Spleen much enlarged, rounded edges, tough. Liver very pale, mottled with yellow. Adrenals very large (left 1.383 gm.; right 1.169 gm. weighed fresh), float in formol solution (10 per cent). Kidneys of normal size and consistency, surface smooth, yellow striations in pyramid following the normal rays; here and there, at base of pyramid, small yellowish nodules. Bilateral purulent pleurisy. Discrete patches of consolidation in left lung. Frozen sections of aorta show the intima to be *twice* as thick as the muscular coat. Histological details are omitted because they add nothing to the present knowledge of this experimental condition.

D 12.—Female, white rabbit. Dec. 10, 1923, left carotid loop is made; weight 1.855 kilos. Jan. 7, 1924, first blood pressure readings; weight 1.970 kilos.

Feb. 26, egg yolk feeding is started; weight 2.435 kilos (one egg yolk in bread, daily).

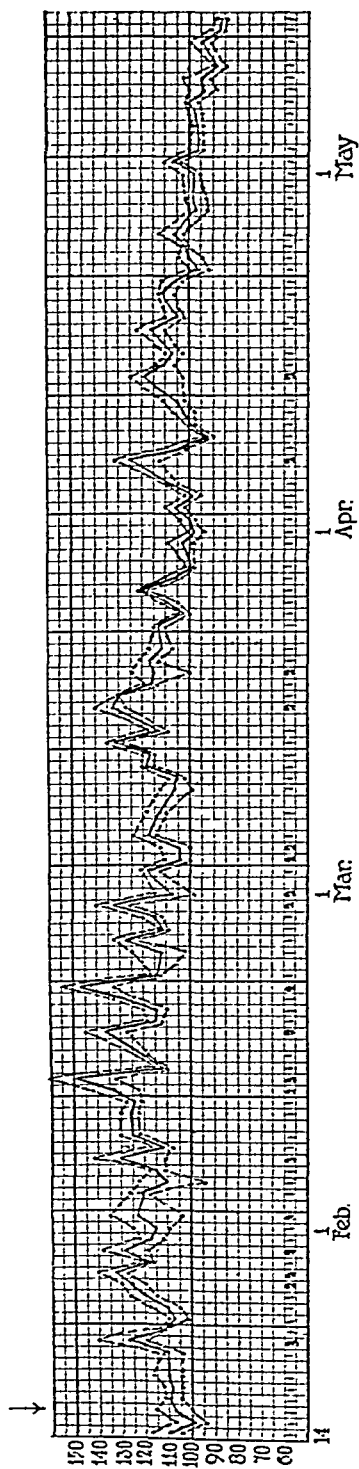
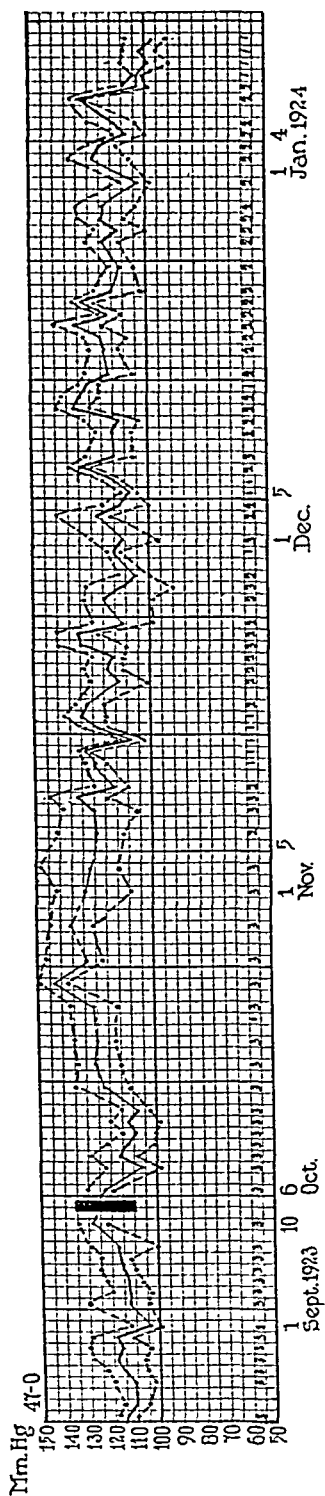


Fig. 1.

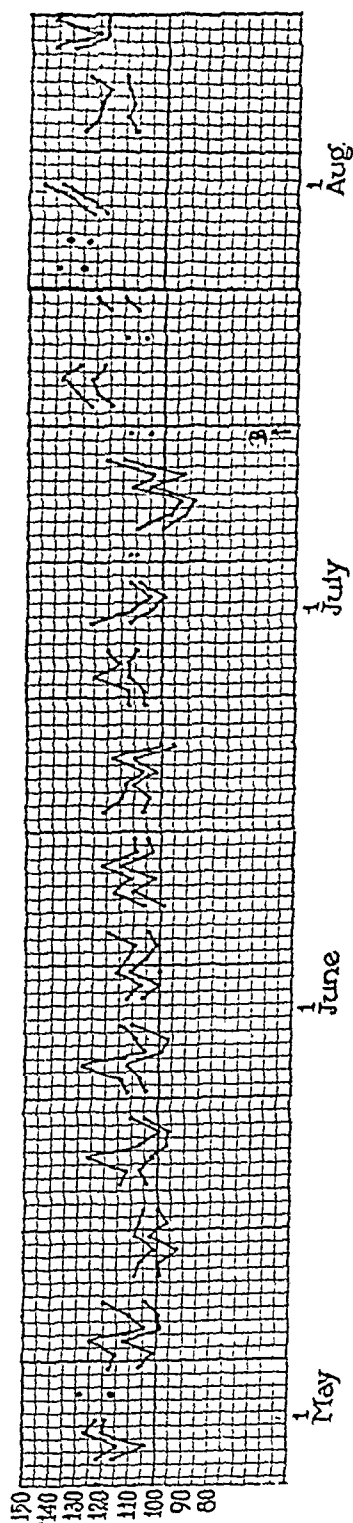
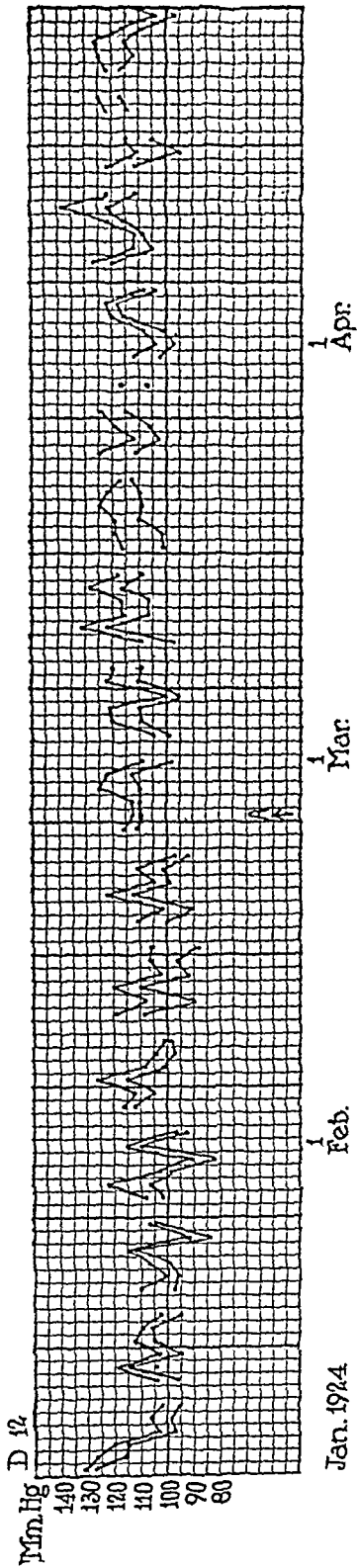


FIG. 2.

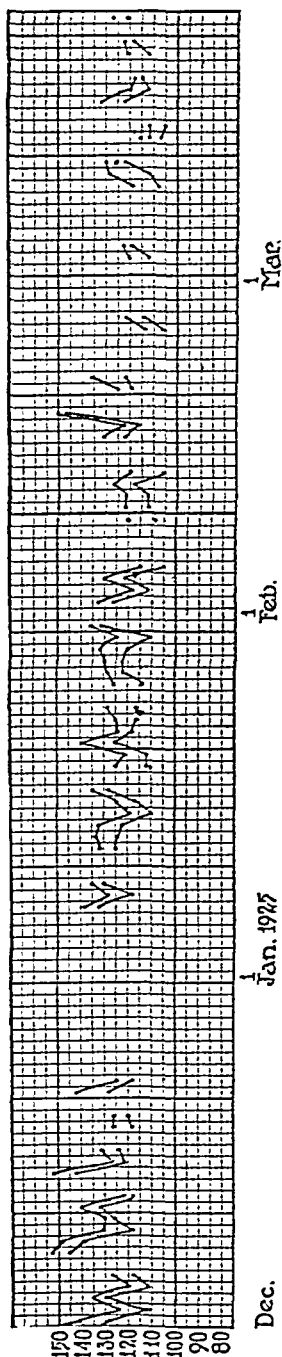
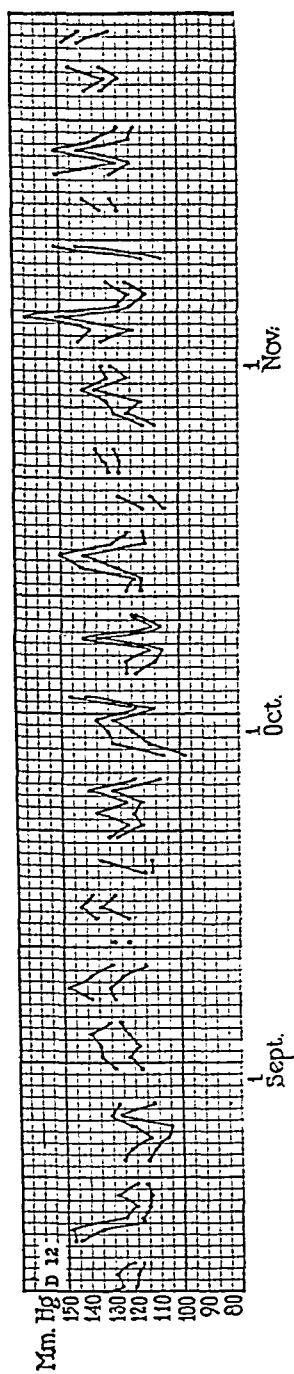


FIG. 3.

Apr. 30, egg yolk in milk, through stomach tube. May 1, egg yolk in water, stomach tube, daily. Weight 2.615 kilos.

July 14, two egg yolks in water given, daily. Dec. 27, weight 3.335 kilos. Mar. 17, 1925, weight 3.220 kilos.

Mar. 23, animal in excellent condition. It was sacrificed (chloroform) because the experiment was considered to have lasted sufficiently long. The animal did not receive any eggs from Dec. 28, 1924, to Jan. 5, 1925, inclusive. Occasional days scattered throughout the experiment were missed also.

Total number of egg yolks consumed, 531.

For *blood pressure* see discussion. Graph is reproduced in Figs. 2 and 3.

*Autopsy.*—*Aorta* and *pulmonary* artery are opaque, thick. Intima is rough, yellowish, wrinkled. In the pulmonary artery the infiltration is greatest around the bifurcation and extends into its branches. In the aorta the infiltration begins at the ring and extends clear down to the iliacs. All the main trunks are infiltrated a short distance from their root. The arch is dilated, and here the infiltration is greatest. Carotids and iliacs are not infiltrated. Mitral valve is thoroughly infiltrated; tricuspid only slightly. Liver: intense fatty infiltration. Costal margin has made a deep impression on convex surface and in this area the infiltration is greatest. Spleen somewhat enlarged. Transverse colon possesses a distinct yellowish discoloration of the mucosa. Uteri are enlarged, and vagina is greatly dilated (contains abundant mucus); cervixes, full of clear, transparent polypi. Uterine mucosa is full of like polypi. Adrenals are large, left weighs 570 mg., right 471 mg., weighed fresh. Kidneys normal in size and shape; surface moderately pitted; cut surface shows a few well defined, large yellow nodules in the boundary zone, somewhat spindle-shaped, with the long axis parallel to the medullary rays; besides these large fatty deposits there are fine yellowish striæ in the medulla.

#### DISCUSSION.

*Autopsy Findings.*—All the animals developed varying degrees of atherosclerosis of the aorta; from a few flat clusters scattered here and there (D 1) to a total infiltration from root to bifurcation (No. 47-0 and D 12). The most severe lesion was found in No. 47-0 (117 egg yolks) where the fatty intima formed two-thirds of the total thickness of the aorta (7). D 6, after 210 egg yolks, and D 12, after 531 yolks, had a

less profound infiltration, although it extended to the whole length of the aorta in D 12. The least infiltration occurred in D 1, which received the smallest number of eggs (40 eggs). The pulmonary artery was involved in all, the most extensive infiltration occurring again in No. 47-0.

The largest corneal infiltration was seen in D 6, a brown female rabbit. Corneal arcs were absent in D 10 (55 eggs) and D 12 (531 eggs). The former was a brown female rabbit, the latter an albino, female also. Schönheimer (8) concludes, from his experiments, that males are more resistant than females as far as the production of corneal arcs is concerned, and that extensive arcs are obtained only in females after prolonged administration of cholesterol. In accordance with this, the most extensive corneal infiltration was present in a female, but another female had no corneal arcs after 531 eggs. Since this animal was the only albino of the group, and Schönheimer says nothing about the color of the rabbits, this observation should be emphasized. On the other hand, D 10 had no corneal arcs after 55 egg yolks, while D 1, a brown male, had good corneal arcs after 40 eggs.

The fatty infiltration of the liver was most intense in D 12, without any evidence of a cirrhotic process; but in D 6 the liver was firm and in some places distinctly coarse. The spleen was considerably enlarged only in one (No. 47-0). The kidneys showed fatty streaks in the pyramid in three (D 10, No. 47-0, D 12) and discrete clumps in the boundary zone (Bailey) in two (No. 47-0 and D 12).

The adrenals were of good size in all. Unfortunately some of the adrenals were not weighed fresh, but after several years standing in formaldehyde solution. To get an approximate idea as to the effect of such prolonged fixation on the weight, the adrenals of No. 47-0 and D 12, which had been weighed at the time of the autopsy, were weighed again, that is, after an almost equally long formol fixation, with the result that the left gland of No. 47-0 *gained* about 8 per cent (the right had been sectioned and a piece removed for microscopic examination) whereas the adrenals of D 12 *lost* about 4 per cent. If it be assumed that all the other adrenals *gained* 8 per cent (to assume the worst case) and this amount be subtracted from the recorded weight, an average of 667 mg. for the left and somewhat higher for the right is obtained,



far greater than the corresponding averages 238 and 221 mg. for fourteen normal rabbits above 2 kilos in body weight and greater than the averages 353 and 341 mg. for fourteen rabbits above 2 kilos in body weight, whose thyroid and parathyroids had been removed by Marine (Stewart and Rogoff (9)). In the extensive statistical work of Brown, Pearce and Van Allen (10) on 645 normal *male* rabbits, mainly from eastern Pennsylvania and the immediate vicinity of New York City, the mean combined weight of the adrenals is given as 0.383 gm. More details are unnecessary, since all these findings have been described before (8). My sole purpose is to show that the present animals actually had the now well recognized picture of experimental cholesteatosis, and that in particular the aorta showed from slight to extreme fatty intimal deposits. None of these aortas showed any gross calcification of the media. Schönheimer observed a marked calcification of the thoracic aorta in one of his animals, and says, very naively, that it is probably not due to the diet, but rather to the use of the stomach tube, which through repeated, short elevations of the blood pressure, may act like adrenalin injections. Although he had Schmidtmann's method (11) at hand, he did not use it to see if there was really an elevation of blood pressure, which if found, could still be ascribed to struggle or excitement, since the animals do not take the tube without resisting, and granting, of course, that adrenalin necrosis is mechanically produced and not due to a toxic action or something else.

The kidney deserves special mention. Bailey has found it more frequently affected than other investigators (Schönheimer). Bailey found the surface pitted in four out of nine animals egg-fed, or in six out of eight whose kidney showed gross cholesterol lesions. I find striæ and nodules also, but scars in the cortex only in one (D 12), a moderate scarring in fact, and although this animal received nine times as many eggs in less than five times as many days as Bailey's Rabbit 7, the xanthomatose lesions appear insignificant when compared with the extraordinary lesions illustrated in Bailey's paper (his Fig. 7, kidney of Rabbit 7). There were no scars in the kidneys of D1 and D10 which received lead carbonate. I am inclined to believe that these xanthomatose formations are secondary to a preexisting scarring of the cortex, a view considered by Bailey himself and by Schönheimer.

*Blood Pressure.*—Fig. 1 contains *all* the essential data from No. 47-0. The lower broken line represents the lowest reading of the corresponding day, the upper broken line the highest reading. The solid line is the calculated arithmetic mean. No figure has been discarded, but the measurements taken from July 31 to August 23 have been omitted to shorten the graph. The range of pressure in the omitted period covers from 90 mm. to 134 mm. Hg, with a mean of 110.0 (420 readings). The small figures at bottom of the graph multiplied by 10 give the number of readings of each day. No readings were taken from September 11 to October 5 (black bar in graph), or on isolated days (break in sequence of bottom figures). Egg feeding was started on January 16 (arrow in graph). The only difference in the blood pressure curve before and during the egg feeding consists in the wider range of daily oscillations in the former, a difference readily accounted for by the larger number of daily readings. Toward the end, when the animal was obviously sick (see protocol), the blood pressure was low. The blood pressure reached about 150 on two occasions, both before and during egg feeding. The details of these days are as follows:

Oct. 24, 1923, 10.41 a.m. 145-144-146-144-147-143-144-142-141-141 (pulse rate 200).

10.49 a.m. 149-145-143-144-141-143-145-142-143-146 (pulse rate 160).

10.58 a.m. 144-146-142-142-139-137-137-140-139-138 (pulse rate 160).

Nov. 3, 1923, 3.45 p.m. 129-127-126-126 = 127-126-130-130 = 130-130 (pulse rate 200).

3.51 p.m. 146-148-146 = †128-120-115-116 = 116-116-116 (pulse rate 184).

At the point indicated by † animal moved backward, in box.

3.58 p.m. 129-130-129-128 = 121-120-123-125 = 121-123 (pulse rate 176).

Feb. 14, 1924, 12.08 p.m. 160-160-155-153 = 156-150-152-150 = 150-148-147-152 = 152-147-146-142 = 149-152-150-145 (pulse rate 200). Mouth piece removed for a few seconds, cuff in place, animal did not move.

12.17 p.m. 145-142-135-132 = 139-140-149-140 = 144-146 (pulse rate 188).

4.59 p.m. 118-121-122-122 = 128-129-129-131 = 136-134 (pulse rate 184).

5.07 p.m. 155-153-152-150 = 145-144-144-142 = 143-144  
(pulse rate 180).

Feb. 22, 1924, 3.18 p.m. 138-133-139-139 = 142-142-145-143 = 150-150-  
149-144 = 149-150-150-149 = 150-153-153 =  
153 (pulse rate 176).

Pulse rate behaved as follows:

Fastest, Aug. 30, 1923, 248 per minute, with a blood pressure between 125 and 130 mm. Hg, mean 126.7 (first 10 readings).

Slowest, Nov. 27, 1923, 128 per minute, blood pressure 90-104, mean 95.3 (1st 10 readings).

Apr. 19, 1924, 128 per minute, blood pressure 102-110, mean 104.8 (10 readings).

Apr. 30, 120 per minute, blood pressure 94-102, mean 98.3 (10 readings).

May 1, 128 per minute, blood pressure 95-102, mean 98.2 (10 readings).

On May 12, 13 and 14, when the animal was suffering from a pleuropneumonia, the pulse rate was 192, 224, 192, respectively, and the blood pressure oscillated between 84 and 100 mm. Hg. The curve illustrates one of those rare animals which cover the whole range from 90 to 150 mm. Hg, that is, almost the totality of the normal fluctuations of blood pressure in the rabbit. Without special indication it would be impossible to pick out the egg feeding period. D 1 with a maximum pressure of 135 mm. Hg and D 6 with a maximum of 143, may be dismissed without further discussion. D 10 does not differ essentially from No. 47-0.

There remains D 12, whose graph is reproduced in Figs. 2 and 3. During January and February, 1924, up to *A* (see Fig. 2), blood pressure oscillated between 83 and 132 mm. Hg, with a mean of 104.9. From *A* to *B*, 139 days (animal receiving one egg yolk daily), the blood pressure oscillated between 88 and 138 mm. Hg, with a mean of 109.4, the oscillations being somewhat greater during March and April than during May and June. From *B* on to the end of the experiment (animal receiving two egg yolks daily), the blood pressure was in general higher and the oscillations became larger, between 98 and 165 mm. Hg, with a mean of 124.0. Pulse rate behaved as follows:

Fastest, Nov. 5, 1924 (resistance), 256 per minute, blood pressure 151-164 mm. Hg, mean 155 (20 readings).

Nov. 17, 256 per minute, blood pressure 128-151, mean 135.3 (20 readings).

Slowest, June 3, 1924, 132 per minute, slightly arrhythmic, blood pressure 100-107 mm. Hg, mean 102.1 (10 readings).

July 9, 1924, 136 per minute, blood pressure 89-94, mean 91.4 (10 readings).

The protocols of the days of highest blood pressure are as follows:

Nov. 5, 1924, resistance. 2.32 p.m. 164-164-162 = 160-158-157-155 = 154-154-153-151 = 152-152-151-151 = 153-152-153-153 = 152 (pulse rate 256).

Dec. 1, 1924, 10.25 a.m. 165-163-160-155 = 152-153-149-150 = 145-142-142-142 = 142-142-141-139 = 138-140-139-135 (pulse rate 200).

The curve from *B* on to the end is representative of the average blood pressure of a good number of the normal rabbits. It compares well also with the curve of D 46 (Fig. 6, in a previous paper (5)) before and after double adrenalectomy, but oscillates about a lower level than D 65 (Fig. 4, in the same paper (5)), also adrenalectomized. Taken alone, this part of the curve has, therefore, little interest. It gains interest, only when brought into relation with the first part of the curve, and particularly when it is remembered that from *B* on two eggs were given daily instead of one. But, interesting as it may be, is it significant? The answer may be split in two according to the meaning to be attached to the word "significant." If by "significant" is understood "fluctuations beyond the range of blood pressure of normal rabbits in general" the answer is immediate and negative, as appears clearly enough from my former work (3, 4) and from the preceding paragraphs, or from another glance at the normal curve of No. 47-0 (Fig. 1). If by "significant" is meant "fluctuations within the normal range, but high relatively to the fluctuations of the pressure in the same animal during a period of observation, more or less short, before the experiment," (and these constitute the large majority of the claims found in the literature), then the answer is very difficult, perhaps impossible. It was stated at the beginning of this article that there is a fallacy in ascribing any rise in blood pressure to the experiment which is being done, because sometimes rises of pressure

without known cause were seen in animals employed for observation for a long time. Moreover, D 12 is the only one of five animals in which such an effect is observed. From *A* to *B* the animal consumed 130 egg yolks and it is highly probable, judging from the other four experiments and from Bailey's experience, that at the end of that time there was already a good infiltration of the aorta. So that from the standpoint of the etiological relation of high blood pressure to the development of atherosclerosis, this observation is not a favorable one, and the other four are no better. Although the pulmonary artery is just as frequently and almost as severely involved as the aorta, I have not seen any report claiming an increased blood pressure in the pulmonary artery of these rabbits. Not knowing how to measure this pressure, I shall not discuss it, but clearly an examination of this fact will have to be considered by those who believe in the mechanical theory of atherosclerosis and especially by those who may find in experimental conditions material for their clinicopathological speculations. Whatever it may be, these experiments, positive as far as the production of atherosclerosis of the aorta is concerned, are far from satisfying the criterion I have given for a pathologic high blood pressure in rabbits (4).

*Analysis of Van Leersum's Work (2).*—Van Leersum does not mention the number of animals examined, or the length of time during which they were examined. He contents himself with saying that with his method of measuring the blood pressure (an excellent method, I believe) he has determined "regularly and for a very long time" the blood pressure oscillations of normal rabbits and of rabbits subjected to liver feeding. But there is no explicit mention of the actual oscillations found.

He says that all values were recorded, but the first ones usually were not taken into account; that only a series of 5 values which did not differ very much from one another were considered admissible (page 416), but this procedure is arbitrary and Van Leersum does not justify it. The criterion which guided him is as follows: "Die im Anfang erhaltenen Werthe jedoch sind wegen der Unruhe des Thieres in der Regel weniger gleichmässig: der Blutdruck ist dann oft bedeutend erhöht oder ermässigt. Während des Messens kommen die Thiere aber allmählich zur Ruhe und wird der Blutdruck gleichmässiger." For instance, on page 417, where a sample of a protocol is given, 11 figures (9 a.m.) are discarded, from 182 to 205 cm. water, and 5 retained, from 195 to 199 (average 196); at 2 p.m., 5 figures are discarded, from 163 to 170, and the average of the retained figures is 159. It results from all this, that Van Leersum's curves do not repre-

sent the fluctuations of blood pressure of his animals, but are constructed from sets arbitrarily selected among a wide range of values.

He reports the blood pressure of eight animals to which he gave powdered liver and of four more to which he gave sodium taurocholate or sodium glycocholate.

The summary of his results is as follows: No. 87, "average" blood pressure during normal feeding (1 week), 181.6 cm. water; during liver feeding (another week), 194.5 cm. water. No. 57 was given liver every other week. The values (means) as they appear in the graph (Curve 1) are as follows, normal feeding weeks being placed in parentheses: (163) 158 (152) 164 (156) 156 (144) 164 (162) 173 (159) 162 (156) 158. With the exception of 173, which occurs during the liver feeding, all the others are equal to or less than the first figure 163, which happens during a normal period; for differences of 1 cm. of water (less than 1 mm. of Hg) lie within the experimental error. The instance in which the average blood pressure was 173 is less than the average blood pressure of No. 87 during the control period. Rabbit F, early in October, showed an average of 164 cm. (1 week), and in the 1st week of November, still under normal feeding, 180 cm. In the 1st week of liver feeding the average blood pressure was 173, and in the 4th week 181 cm. Rabbit G, early in October, had an average blood pressure of 162.5 cm.; in the 1st week of November, 169 cm. At this time the liver feeding was started. In the 3rd week of this feeding the pressure averaged 171 cm., and in the 6th week 189 cm. water. Van Leersum adds: "Eine Steigung also von gut 11 pCt." But Rabbit F, just mentioned, jumped from 164 to 180 cm., an increase of 10 per cent, without the help of liver feeding. The other four animals may be considered jointly (A, C, D, 23). The averages during the normal feeding period varied between 141 (D) and 172 (C), the observation lasting 1 week for C, 2 for A and D, and irregularly in July, Sept., and Dec., 1911, for No. 23. The maximum weekly average recorded, *during a diet period*, appears as follows: 206 (A), 219 (C), 216 (D), 198 (23) and *after a liver feeding*, 221 (D), in the week following the cessation of the abnormal nourishment.

Van Leersum discusses these findings in the next 3 pages and then comes to the question of what part of the liver is responsible for this effect on the blood pressure. He tries the bile salts (sodium taurocholate and sodium glycocholate) and says: "ihre Wirkung auf den Circulationsapparat ist eine lähmende und sie vermindern den Blutdruck, wie Versuche an vier Kaninchen mich gelehrt haben, in erheblichem Maasse." The protocols are brief but very instructive. I copy from them the pertinent figures. Blood pressure during control period, average, Rabbit Q, 200 cm. water; Rabbit Z, 221; Rabbit X, 208; Rabbit Y, 220. Blood pressure after about 1 month of bile salt (mixed with the food, carrot), average, Rabbit Q, 168 cm. water; Rabbit Z, 190; Rabbit X, 161; Rabbit Y, 182. In other words, figures like 161, which in the main part of the paper are considered normal, are now interpreted as due to the injurious effect of the bile salts, and figures between 200 and 221 which were interpreted before (Rabbits A, C, D, 28) as due to the effect of liver feeding are now considered

normal. If Van Leersum had examined more normal animals and for a longer time, before any experiment was undertaken, he would have interpreted his results differently. I venture this statement because my figures for normal animals include *all* of Van Leersum's figures. 122 cm. water (Curve 6, Nov. 2) and 239 cm. water (Curve 2, Jan. 10) are the lowest and highest figures, respectively, recorded in the papers under discussion, that is, about 90 mm. and 176 mm. Hg respectively. In Fig. 2, Rabbit 489, Graph IV of a previous paper (3), there may be seen a few instances of pressure about 180 (averages, since Van Leersum's figures are averages also) and in Figs. 3 and 4, Rabbit 483, Graphs I, II, and III, there are several below 90.

The analysis may be summarized thus: (1) Van Leersum's range for the normal blood pressure in the rabbit, as recorded by his method, is confirmed; (2) Van Leersum's conclusion concerning the influence of a liver diet on the blood pressure of the rabbit is not substantiated by his data, since the fluctuations of blood pressure he obtained do not surpass his own recorded figures for normal animals.

*Note on the Results Obtained by Other Methods.*—The work of Fahr, Schmidt-mann and Schönheimer, together with an account of the methods used by these authors to measure the blood pressure, has been summarized by Shapiro and Seecof (12), who in their own experiments used Anderson's method. I have answered their criticism of Van Leersum's method elsewhere (4). A few remarks, however, may not be out of place here, particularly since a few reports have appeared subsequently to Shapiro and Seecof's.

1. Van Eweyk and Schmidtmann (11) state that the blood pressure of a healthy rabbit, as recorded by their method, lies between 90 and 100 mm. of Hg. They do not say how many animals they have examined or for how long a period. By comparing their method with the values obtained with a Cowl-Gad's manometer, they find an agreement between their figures and the minimal pressure of the "blutige" measurement. But they add: "Zunächst lassen wir es dahingestellt sein, ob diese Übereinstimmung gesetzmäßig ist oder nicht." In Schmidtmann's first report (13) (six rabbits) the blood pressure was taken *once weekly*. The lowest figure is 88 (Protocol IV) before the experiment, the highest 132 (Protocol VI) in the 2nd and 3rd weeks of liver feeding. In her second report (14), she speaks of feeding experiments (diets rich in cholesterol) on 67 rabbits: in some the blood pressure effect was negative, in the large majority, however, she states that the pressure rose to 120–140 mm. Hg for weeks and even months. Then the blood pressure fell to normal, whereas the blood cholesterol remained high. She explains this fall by assuming an injurious effect of cholesterol on the heart muscle and the vascular system. It is difficult to interpret these data because it is not at all clear what is being measured with that method. The authors think that their figures agree with the minimal pressure of the aorta, but have left "undecided" (their words) how legitimate this agreement is. On the other hand, *one* measurement *once* a week gives entirely too little information on the blood pressure of the rabbit, to draw positive conclusions therefrom.

Schönheimer (8) says that he has also found an increase in blood pressure (Schmidtmann's method) under the influence of cholesterol feeding, but he gives no data, except that the largest increase was from 80 to 112 mm. Hg.

Anitschkow (15) says he has confirmed Schmidtmann's results, but gives no details, not even mentioning the method used.

Deicke (16) reports his findings on 88 rabbits fed on cholesterol or liver. He used Schmidtmann's method, but there is no statement as to how often or how many times the blood pressure was measured. Judging from his graphs, the blood pressure was taken *once a week*, sometimes more than a week apart, sometimes less. The normal curve reproduced shows fluctuations between 96 and 110 mm. The highest values represented in his graphs are, in mm. Hg, 131 (Curve 5, liver feeding), 132 (Curve 6, after intravenous injection of cholesterol solution), 133 (Curve 3, enteral cholesterol), 142 (Curve 2, enteral cholesterol).

Thölldte (17) states that the blood pressure of the rabbit, taken *twice daily* for months (Schmidtmann's method, cholesterol feeding) shows no increase "beyond the physiological fluctuations." Unfortunately he gives no details or figures, so that it is not known what is meant by physiological fluctuations.

2. Anderson's method (18) consists in the recording of the pressure necessary to obliterate the pulsation of one of the ear arteries of the rabbit. The greatest error comes, according to Anderson, from the changes in the caliber of the vessel, but he says that they can be largely controlled by keeping the ears warm. The blood pressure, in his normal animals, ranges between 75 and 90 mm. Hg. That the conditions laid down for the measurements must be closely adhered to is obvious from the recent report of Behrens (19), who devised a method essentially the same as Anderson's, and says that the values obtained in this fashion are very constant and lie about 40 mm. Hg. He does not mention Anderson's work, but quotes the work of Kuraya (20), who, independently of him, designed the same method of blood pressure measurements and obtained the same values (mean blood pressure in healthy animals 35-50 mm. Hg). Shapiro and Seecof (12) used Anderson's method and concluded that "the systolic blood pressure of the central artery of the rabbit's ear averages between 75 and 90 mm. Hg as reported by Anderson." In their table the figures for the controls are not essentially different, ranging between 77 and 105 mm. Hg. They fed lanolin to rabbits which had been subjected to various surgical operations (splenectomy, thyroidectomy, double adrenalectomy and combinations of double adrenalectomy with splenectomy or thyroidectomy). The number of blood pressure readings was very small: from 2 to 6 during the whole course of the experiments. The conclusion reached was that there is no significant hypertension during the developmental stage of experimental lanolin atherosclerosis in rabbits.

#### SUMMARY.

Egg yolk was fed to five rabbits provided with a good carotid loop (Van Leersum's method). The blood pressure was measured daily



before and during the egg feeding until the animal's death. The duration of the experiment varied from 81 to 391 days. The number of eggs consumed varied from 40 to 531. Two animals received, in addition to the egg yolk, lead carbonate by mouth.

Autopsy findings, blood pressure readings and pulse rate are discussed. Two blood pressure graphs are reproduced. The paper contains a brief analysis of Van Leersum's work on liver feeding and a review of the results obtained by other methods of measuring the blood pressure in the rabbit.

#### CONCLUSIONS.

1. Van Leersum's range for the normal blood pressure in the rabbit, as recorded by his method, is confirmed.

2. Van Leersum's conclusion concerning the influence of a liver diet on the blood pressure of the rabbit is not substantiated by his data, since the fluctuations of blood pressure he obtained do not surpass his own recorded figures for normal animals.

3. Fluctuations of systolic blood pressure beyond the "normal" range are not *necessary* for the production of experimental atherosclerosis of the aorta in rabbits. Inversely, egg yolk feeding experiments in rabbits in which atherosclerosis of varying degree, even extreme, is obtained, are not accompanied by an elevation of blood pressure *outside* the "normal" range.

4. The fluctuations of blood pressure observed during experimental atherosclerosis do not simulate the condition of essential hypertension in man.

I am indebted to Dr. G. N. Stewart for guidance and inspiration in the course of this work.

#### BIBLIOGRAPHY.

1. Fahr, T., *Verhandl. deutsch. path. Ges.*, Jena, 1912, xv, 234.
2. Van Leersum, E. C., *Z. exp. Path. u. Therap.*, 1912, xi, 408.
3. Dominguez, R., *J. Metabol. Research*, 1924, vi, 123.
4. Dominguez, R., *J. Exp. Med.*, 1927, xlvi, 443.
5. Rogoff, J. M., and Dominguez, R., *J. Metabol. Research*, 1924, vi, 141.
6. Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P., *Medicine*, 1925, iv, 1.
7. Bailey, C. H., *J. Exp. Med.*, 1916, xxiii, 69.

8. Schönheimer, R., *Virchows Arch. path. Anat.*, 1924, ccxlix, 1.
9. Stewart, G. N., and Rogoff, J. M., *Am. J. Physiol.*, 1921, lvi, 220.
10. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1926, xliii, 733.
11. van Eweyk, C., and Schmidtman, M., *Virchows Arch. path. Anat.*, 1922, ccxxxvi, 420.
12. Shapiro, S., and Seecof, D. P., *J. Lab. and Clin. Med.*, 1925, x, 826.
13. Schmidtman, M., *Virchows Arch. path. Anat.*, 1922, ccxxxvii, 1.
14. Schmidtman, M., *Verhandl. deutsch. path. Ges.*, Würzburg, 1925, 118.
15. Anitschkow, N., *Virchows Arch. path. Anat.*, 1924, ccxlix, 73.
16. Deicke, O., *Krankheits Forsch.*, 1926, iii, 399.
17. Thölldte, *Centr. allg. Path. u. path. Anat.*, 1926, xxxviii, 590.
18. Anderson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1923, xx, 295.
19. Behrens, A., *Arch. ges. Physiol.*, 1926, ccxii, 372.
20. Kuraya, *Acta Schol. Med. Univ. Imp. Kioto*, 1924, vi, 373, cited by Behrens (19).



# STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

## VII. ALLERGIC REACTIONS WITH STRAINS FROM ERYSIPELAS.

By A. R. DOCHEZ, M.D., AND FRANKLIN A. STEVENS, M.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, Presbyterian Hospital, New York.)

(Received for publication, June 10, 1927.)

Dochez and Sherman (1) have recently described sensitization reactions which could be neutralized by immune serum. These reactions were obtained in guinea pigs and rabbits which had been previously inoculated with living cultures of *Streptococcus scarlatinæ* or with filtrates of this streptococcus. About 10 days after the inoculation, intracutaneous injections of filtrate of cultures of scarlatinal streptococcus caused local edema and erythema. The reaction was not obtained with filtrate which had been heated or with mixtures of filtrate and scarlatinal immune serum.

Some strains of hemolytic streptococcus recovered from erysipelas produce toxic substances similar in certain respects to the substances produced by strains from scarlet fever. Cutaneous reactions may be obtained in man with the filtrates of the strains from erysipelas. The reactions obtained with a majority of the filtrates from these strains are not neutralized by scarlatinal sera but may be neutralized with sera prepared with erysipelas strains. Having this similarity in mind, we have studied the cutaneous reactions in rabbits during immunization with filtrates of strains from erysipelas.

### *Methods and Experimental Data.*

Two strains of hemolytic streptococcus, EA and EM, were selected for the immunization of the rabbits employed in these experiments. One strain was obtained from facial erysipelas, and the second from a blood culture on a patient with phlebitis. Filtrates from cultures of both strains caused local erythema when injected intracutaneously in children and adults. The strongest reactions were obtained in children with filtrates from 48 hour cultures in tryptic digest broth.

Reactions were not obtained with filtrate which had been heated 2 hours in the Arnold sterilizer. Scarlatinal antitoxin added to the filtrates before injection did not modify the size or intensity of the erythema following intracutaneous inoculation, but the serum from rabbits immunized with erysipelas filtrates completely neutralized some of the reactions occurring with a 1/250 dilution of the filtrate.

A number of normal rabbits were tested with 0.1 cc. of undiluted filtrate before sensitization. About 20 per cent showed an indefinite erythema at the site of the intracutaneous inoculation, but none of the reactions observed at this time was as intense as the mildest reactions occurring later during immunization. Following these preliminary tests, six of the rabbits were inoculated intracutaneously with 1.0 cc. of undiluted filtrate at intervals of about 12 days. On the day preceding each inoculation skin tests were done with 0.1 cc. of the undiluted filtrates of cultures of the two strains EA and EM. Control tests were done with filtrate heated 2 hours at 98°C.

After the third inoculation edematous reactions were observed in four of the six rabbits at the site of the cutaneous injection of the filtrate. Edema and erythema occurred about 24 hours after 0.1 cc. of the undiluted filtrate was injected into the skin. This erythema lasted approximately 48 hours. The first positive reactions were observed about the 30th day after immunization was begun and occurred only with unheated filtrate. At this time the heated filtrates did not cause reactions. After the fourth inoculation erythema and some edema occurred with the heated as well as the unheated filtrate. After the fifth inoculation all the reactions were less intense than those previously observed and after the 57th day strongly positive reactions were no longer obtained. Two rabbits showed no sensitization during this period of observation.

Intracutaneous inoculations of 1.0 cc. of filtrate were continued until seven or eight injections had been given. During the interval between the time that the animals last showed cutaneous sensitization (approximately the 57th day) and the final intracutaneous inoculation two of the rabbits showed a transient recrudescence of activity. During this recrudescence the reactions obtained were exceedingly mild. After the seventh or eighth intracutaneous inoculations which were given between the 80th and the 100th days, filtrates were injected intravenously. 5 cc. and later 10 cc. were given at intervals of 2 weeks over a period of 3 months.

On the 187th day after the first intracutaneous inoculations, when the animals had been immunized for 3 months intracutaneously and 3 months intravenously, cutaneous reactions were again done. At this time all the rabbits, including the two which had previously shown no cutaneous hypersusceptibility reacted strongly with the unheated and heated erysipelas filtrates and with a filtrate of a scarlatinal streptococcus. Tests were also done at this time with filtrates of a strain of hemolytic streptococcus from a normal throat and with broth. Only one of five rabbits reacted with the throat strain. Two of the five gave questionable reactions with broth. None of the reactions was neutralized by the addition of scarlatinal or erysipelas immune sera to the filtrates previous to the injection. Serum was

*Cutaneous Allergic Reactions with Filtrates of Strains of Hemolytic Streptococcus from Erysipelus during the Immunization of Rabbits with Filtrates of Erysipelus Strains.*

Intervals days	Rabbit I				Rabbit II				Rabbit VI				Rabbit X				Rabbit XIV				Rabbit XVI			
	Cutaneous reactions				Cutaneous reactions				Cutaneous reactions				Cutaneous reactions				Cutaneous reactions				Cutaneous reactions			
	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated	Filtrate A unheated	Filtrate A heated	Filtrate M unheated	Filtrate M heated
1 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
46 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
57 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
68 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
78 m	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
92	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
115 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
123 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
136 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
153 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
176 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
187	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
210	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Six rabbits were immunized with filtrates of two strains of hemolytic streptococcus from erysipelas. Filtrates of these strains have been designated by the letters A and M corresponding to the strain with which the filtrates were prepared. The rabbits were first inoculated intracutaneously and later intravenously at the intervals indicated in the first column on the left. Intracutaneous filtrate and intravenous filtrate are indicated by small and capital letters respectively in this table. Inoculations followed by an asterisk were given only approximately at the interval indicated in the first column. Preceding each inoculation cutaneous reactions were done with heated and unheated filtrates A and M. Each rabbit was tested with these four preparations. The reactions resulting from these intracutaneous tests are indicated by plus signs. Plus signs in heavy type have been used for edematous reactions.

obtained from each animal at this time and later tested for neutralizing substances in a second series of hypersusceptible animals. After the serum was obtained, desensitization was attempted with filtrates of scarlatinal and erysipelas strains, and with broth. 10 cc. of filtrate or broth were given intravenously 24 hours previous to intracutaneous tests. Desensitization occurred with the streptococcal filtrates but not with broth.

TABLE II.

*The Neutralization of Cutaneous Allergic Reactions in Rabbits Sensitized with Filtrates of Strains of Hemolytic Streptococcus from Erysipelas.*

Sensitized rabbit tested	Reaction with 0.1 cc. of Filtrate M	Filtrate M and Serum I		Filtrate M and Serum II		Filtrate M and Serum X		Filtrate M and Serum XIV		Filtrate M and Serum XVI	
		Mixture a	Mixture b	Mixture a	Mixture b	Mixture a	Mixture b	Mixture a	Mixture b	Mixture a	Mixture b
28	++++	+++	+	+++	-	++++	+	++++	-	+++	±
27	+++	++	±	-	-	++++	±	+++	-	+++	-
26	++	+	-	-	-	++	-	++	-	++	-
25	+	+	±	-	±	+	-	+	-	-	±
29	+	±	-	-	-	±	-	±	-	-	-
24	+	+	-	-	-	+	-	±	-	-	-

Six rabbits were sensitized with filtrates of strains of hemolytic streptococcus from erysipelas. The numbers of these rabbits are given in the first column. When the rabbits first showed cutaneous reactions with 0.1 cc. of erysipelas filtrate, neutralization of these reactions was attempted by the addition of immune erysipelas sera obtained from rabbits immunized by intracutaneous and intravenous injections of filtrate from erysipelas strains to the filtrate previous to injection. The sera of five immunized rabbits (Table I) were tested for neutralizing qualities. Each serum was added to filtrate of Strain M. Two proportions of serum and filtrate were employed, Mixture a (serum one part and filtrate four parts) and Mixture b (serum and filtrate equal parts). The rabbits showed no reaction with heated filtrate at the time the neutralization tests were done. Edematous reactions have been indicated in heavy type.

The sera obtained from five of these immunized rabbits were tested for neutralizing substances in a second series of sensitized rabbits. Twenty rabbits were given intracutaneous injections of erysipelas filtrate at weekly intervals until they were sensitive to cutaneous inoculations with 0.1 cc. of the filtrate. Only eight of the twenty rabbits became allergic. The neutralization tests were carried out as soon as allergy developed. Two mixtures of each serum and the filtrate—one part of serum and four of filtrate, and equal parts of serum and filtrate—were pre-

pared and incubated 3 hours. Each of the allergic rabbits was then tested with the two proportionate mixtures of each of the five sera and the filtrate. The amounts of the mixtures used were so graduated that 0.1 cc. of filtrate was injected. Although neutralization occurred with both proportions of sera and filtrate, it was most complete and uniform with the mixture containing equal parts. The neutralizing properties of the immune sera were not uniform, but no relationship was discerned between the strength of the serum and the degree of hypersusceptibility to filtrate shown by the rabbit from which the serum was obtained.

When these neutralization experiments were carried out, these rabbits reacted with unheated erysipelas filtrate but not with heated. 2 weeks later positive reactions were obtained with heated erysipelas filtrate and with filtrates of scarlatinal strains. At this time neutralization of the reaction by the addition of immune serum to the filtrate was questionable. These animals were desensitized with scarlatinal and erysipelas filtrates intravenously. 10 cc. of broth did not have this desensitizing effect.

The results of these experiments have been tabulated. Table I gives the intervals between the injections of filtrate, the method of inoculation, whether intracutaneous or intravenous, the filtrate employed, and the results of the cutaneous tests. Edematous reactions are indicated by heavy type. The neutralization tests with immune erysipelas sera and filtrate have been arranged in Table II.

#### DISCUSSION.

*Allergic Phases during Immunization.*—The rabbits which we have immunized apparently passed through consecutive periods of sensitization—a negative period from the first inoculation to the appearance of the first positive reactions, a positive allergic phase of approximately 4 weeks' duration, next a prolonged period of inactivity, and finally a second phase during which they showed cutaneous sensitization. The first period of negative reactions need not be discussed; it is observed previous to all hypersusceptible reactions before the development of sensitivity. In the animals studied this period varied from 2 to 4 weeks. The second, third, and fourth periods require discussion.

The second period was one of positive hypersusceptible reactions. This period is divided into an early phase of short duration, during which the reactions were neutralized by the addition of immune serum to the filtrate, and a later phase when the reactions were not neutralized. During the first part of this phase when the reactions were



neutralized reactions occurred only with unboiled erysipelas filtrate. The reactions observed later when they were not neutralized by immune serum were less specific, and could be obtained with heated erysipelas filtrate and with scarlatinal filtrate. The most intense reactions were obtained with the erysipelas filtrates.

The third period was a phase of inactivity of uncertain duration. This third phase began about the 68th day when hypersusceptible reactions were no longer obtained. The transition from the second period to this non-allergic state was gradual. The intensity of the reactions gradually diminished from about the 40th day of immunization until the 68th day when not even the slightest erythema occurred after the intracutaneous injection of filtrate. This gradual fading of hypersusceptible reactions has been observed by Zinsser and Grinnell (2) with streptococcus and by Mackenzie and Woo (3) with pneumococcus. Except for the positive reactions occurring later, this negative phase might be considered a return to the normal non-allergic state, or as Zinsser suggests, a state of immunity to the allergin due to the presence of antibodies.

The fourth phase was a positive phase in which the reactions with heated and unheated erysipelas filtrate and with scarlatinal filtrate were equally intense. This phase differed from the first positive phase in three respects; the reactions could not be neutralized, unheated and heated filtrate gave reactions of equal intensity, and all animals, some of which did not become hypersusceptible during the first allergic period were found hypersusceptible at this later time. During this period some of the animals were sensitive to horse serum.

*Possible Factors Responsible for Allergic Reactions.*—The simplest explanation of the various phases of allergy observed in these experiments is the assumption that the phases are the result of hypersusceptibility to different allergins. Dale and Hartley (4) have shown that when serum albumen and globulin are employed to sensitize animals, the intervals required for sensitization are not identical. This would appear to be a satisfactory explanation for the early and late periods of allergy. The somewhat different character of the reactions observed during the two periods supports this hypothesis.

We have already observed that allergic reactions occurring between the 30th and the 60th days of immunization could be neutralized with immune erysipelas serum up to about the 15th day after positive

cutaneous reactions first occurred. Except for the report of Dochez and Sherman we know of no authenticated instance where cutaneous allergic reactions with bacteria have been neutralized with immune serum. Reactions occurring in animals sensitized with the bodies of tubercle bacillus, staphylococcus, streptococcus, pneumococcus, and the Gram-negative pathogens or with extracts of these bacteria have never been satisfactorily neutralized. The filtrates of streptococci from scarlet fever and erysipelas contain toxic substances which are easily separated from the bacterial cells. In one respect these toxic substances resemble the exotoxins of the *diphtheriæ* and tetanus bacilli in that a satisfactory antitoxic serum can be developed by the immunization of animals. Von Behring observed sensitization to diphtheria toxin in 1893. Since this observation allergy to other bacterial exotoxins has been produced experimentally in animals. In view of these previous instances of allergy to exotoxins, and the neutralization of the cutaneous allergic reactions with immune serum in our experiments it seems possible that these cutaneous reactions are sensitization reactions to the toxic substances in the erysipelas filtrate. It also seems probable that the toxicity of these streptococcus products for man is due largely to a state of hypersensitiveness. The less specific reactions occurring later during this first period of sensitization could not be neutralized. These late reactions appear analogous to the sensitization occurring with the bodies and cell extracts of bacteria.

Experimental evidence at the present time indicates that bacterial antigens are of a composite nature and that two substances at least are linked together in the bacterial cell. Zinsser has found that precipitates from extracts of tubercle bacillus contain a non-protein substance designated the "residue antigen," and a second fraction containing nucleoproteins. These fractions are comparable to the S and P substances which Avery and Heidelberger (5) have obtained from the pneumococcus. The S substance is non-antigenic and incapable of inducing allergy. The P substance is antigenic. Animals immunized with this fraction become allergic, and from past experimental work it is probable that this fraction or some substance intimately associated with it is responsible for the allergic reactions ordinarily observed in animals sensitized with bacteria, or to the protein fractions of bacterial extracts.

Rather than assume that two substances were concerned in the first allergic phase, the reactions might also be explained by assuming that the allergin were a complex substance similar to the SP antigen in pneumococcus. The toxic fractions of streptococcus filtrates would be analogous to the SP combination. Allergic reactions to this toxic antigen would be neutralized by immune sera, while reactions due to the P analog from which the specific toxic S element had been separated would be non-specific and not subject to neutralization. As with pneumococcus antigens, reactions would occur with the SP combination and with the less specific P antigen during the immunization of animals.

*Possible Relationship of Allergic Reaction to the Symptoms of Disease Due to Hemolytic Streptococcus Producing Toxic Substances.*—Bristol (6) has recently drawn attention to the hypothesis that the rash and clinical symptoms of scarlet fever are allergic reactions to the products of hemolytic streptococcus. He has carefully reviewed the literature and summarized the evidence presented previously by other authors having a similar opinion. Our sensitization experiments with streptococcal filtrates afford a possible explanation of the rash of scarlatina on this allergic basis.

Infants apparently do not react to intracutaneous injections of scarlatinal filtrate. In adults negative cutaneous tests have been explained by the presence of circulating antitoxin, but Cooke (7) has found that negative reactions occur in infants whose sera have no neutralizing properties. In view of the allergic reactions which Dochez and Sherman have observed in animals hypersusceptible to *Streptococcus scarlatinæ* and the reactions which we have obtained with strains from erysipelas, we suggest that the positive cutaneous reactions with filtrates of streptococcus containing toxic fractions as well as the rash in scarlet fever are the result of previous sensitization. The development of a natural immunity to toxic fractions of scarlatinal filtrates might be explained satisfactorily by this hypothesis. Infants previous to sensitization would react negatively to the toxic substance in the absence of circulating antibody. After sensitization, positive cutaneous reactions would occur accompanied by a susceptibility to rash in event of infection with *Streptococcus scarlatinæ*. With the development of sufficient circulating antibody the

positive reactions would become negative and the tendency to develop the rash would disappear. In this immune state scarlatina *sine* exanthem might occur.

#### CONCLUSIONS.

Rabbits immunized with filtrates of cultures of hemolytic streptococcus from erysipelas show cutaneous allergy. Two periods of allergy have been observed, an early and a late phase. The earliest reactions occurring in the first period of allergy can be neutralized with erysipelas immune sera.

The rash of scarlet fever and the Dick reaction are apparently allergic reactions to products of *Streptococcus scarlatinæ*.

#### BIBLIOGRAPHY.

1. Dochez, A. R., and Sherman, L., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 282.
2. Zinsser, H., and Grinnell, F. B., *J. Immunol.*, 1925, x, 725.
3. Mackenzie, G. M., and Woo, S. T., *J. Exp. Med.*, 1925, xli, 65.
4. Dale, H. H., and Hartley, P., *Biochem. J.*, 1926, x, 408.
5. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367.
6. Bristol, L. D., *Am. J. Med. Sc.*, 1926, clxvi, 853.
7. Cooke, J., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 314, 315.



## STUDIES ON STREPTOCOCCUS BACTERIOPHAGE.

### I. A POWERFUL LYTIC PRINCIPLE AGAINST HEMOLYTIC STREPTOCOCCI OF ERYSIPELAS ORIGIN.

By GREGORY SHWARTZMAN, M.D.

*(From the Laboratories of the Mount Sinai Hospital, New York.)*

(Received for publication, June 15, 1927.)

#### INTRODUCTION.

The influence of the bacteriophage upon streptococci was studied by several authors. Piorkowski (1) was the first to report a lytic principle against streptococci. He mixed 2 cc. of blood from a patient suffering from subacute bacterial endocarditis with glucose broth and filtered this mixture after 4 days incubation. The filtrate showed lysis of the streptococcus obtained from the blood of the same patient. The lytic principle was, according to Piorkowski, transmissible. The qualitative and quantitative potency of this principle was not studied, or were observations made as to whether the principle had any effect upon other pathogenic streptococci.

McKinley (2) claimed that he was able to obtain a lytic principle against a large group of Gram-negative and Gram-positive cocci, including streptococci. To demonstrate such a principle he filtered a given culture containing anti-b. Shiga phage, reinoculated the filtrate, incubated, and filtered again. This was repeated three or four times in succession. When 5 cc. of such a filtrate, diluted 1:10 with broth, was inoculated with the organism from which it was obtained, it failed to grow. This author did not do any experiments on the properties of this principle; namely, on the qualitative and quantitative potency, formation of resistants under its influence, and regeneration of this principle by the resistants. It would be impossible, therefore, to accept these inhibitory factors as evidence of bacteriophage.

Dutton (3) studied the appearance of colonies of various strains of

streptococcus. A large number of strains contained irregular and "moth eaten" colonies and, therefore, suggested symbiotic existence of bacteriophage. Only one strain had proven itself susceptible to a lytic principle obtained from another strain which failed to grow on transplant. The evidence brought forward suggested the existence of streptococcus bacteriophage of a low order which in the majority of instances is not able to produce the classical picture of the bacteriophage phenomenon.

Further work was reported by Hadley and Dabney (4). These authors were able to obtain a potent bacteriophage for one strain of *Streptococcus faecalis*.

Clark and Clark (5, 6) obtained from activated sludge a bacteriophage which was highly potent against two strains of hemolytic streptococci isolated from rabbit and guinea pig lung infections. They failed to adapt this principle to five strains of human pathogenic streptococci.

It is evident that great difficulties accompany attempts to produce an active lytic principle against streptococci. The isolated positive results with a few strains, only two of which were of human origin (Piorkowski (1) and Dutton (3)), leaves the field open for further investigation. The question therefore arises as to whether streptococci as a genus are susceptible to the bacteriophage phenomenon or whether the few strains of streptococci in which this phenomenon was observed carried a factor, not common to other streptococci as a genus, which enabled them to be affected by the phage. With these considerations in mind the author attempted to obtain an active principle against pathogenic human streptococci. The following plan was adopted for this work:

1. To study the effect of Clark and Clark's "sludge" bacteriophage upon the susceptible strain of rabbit streptococcus in order to determine the properties of this principle, its specificity and relation to the classical bacteriophage phenomenon.

2. To make attempts to obtain a powerful bacteriophage for pathogenic streptococci by means of the Clark and Clark phage as well as by other lytic principles.

3. Should these attempts succeed, to study the properties and specificity of such a bacteriophage.

## EXPERIMENTAL.

*Lytic Principles Tested against Pathogenic Streptococci.*

The following lytic principles tested on various occasions against streptococci will be mentioned in the course of this paper:

*B.H.*—A lytic principle kindly sent to me by Dr. A. Gratia, isolated by him from vaccinia pulp. This principle was potent against numerous strains of *Staphylococcus aureus* and *albus*.

*Anti-B. coli Phages.*—A large series of extremely potent bacteriophages was obtained by the author from stools of various animals. They will be mentioned under the name of the animal from which they were obtained.

*L<sub>12</sub> and C/2.*—*L<sub>12</sub>* represents anti-b. Shiga bacteriophage kindly sent to me by Dr. J. Bronfenbrenner. *C/2* is the same phage but adapted to *B. coli*.

*"Sludge" phage.*—This principle was isolated by Drs. Clark and Clark from activated sludge and kindly sent to me a year ago. It proved itself extremely active against a *Streptococcus hæmolyticus* isolated by the same authors from a rabbit. The susceptible strain will be referred to as "Rb" streptococcus.

*Strains of Streptococci Employed in This Work.*

Numerous strains of hemolytic and green-producing streptococci employed in this work were isolated from various sources in this laboratory. Others were kindly sent to me by Dr. A. W. Williams, Dr. Konrad Birkhaugh, and by the American Type Culture Collection. The nature of these strains will be described as the occasion arises.

## I.

Observations on general properties of "sludge" phage corroborate Clark and Clark's report (6). The following requirements which are necessary to identify this principle with the classical bacteriophage can be fully answered:

1. Lysis can be demonstrated on solid media (in the form of so called "eaten up" areas) and in fluid media. The principle diluted to  $1 \times 10^{-8}$  cc. is still able to produce complete inhibition of growth. After lysis occurs such a culture shows again the full concentration of undiluted lytic principle.

2. Lysed culture eventually gives rise to growth. Such a growth is able to regenerate bacteriophage in turn when transplanted into a fluid medium.

3. Formation of resistants is accomplished by prolonged cultiva-



tion of susceptible streptococci with at first highly diluted and then undiluted lytic principle.\*

4. Immunization of rabbits with lysed cultures brings about formation of antibacteriophage serum.

5. In addition it should be mentioned here that this principle is less stable than *coli* dysentery phages. When "sludge" phage is heated to 58° for  $\frac{1}{2}$  hour its lytic exponent ( $E_L$ ) is reduced from -8 to -3. Control heating of dysentery phage to the same  $t^\circ$  does not affect it whatsoever. The lytic potency is also liable to become greatly reduced on standing in the refrigerator for 2 to 3 weeks.

6. The effect of "sludge" phage on other streptococci was studied in fluid media and observations were made 24 hours and 2 to 3 days later. In the course of 1 year 102 strains of pathogenic streptococci of human origin were tested, as follows: 12 strains of the group of scarlet fever hemolytic streptococci; 18 strains of *Streptococcus erysipellatis*; 3 strains isolated by Tunncliffe from cases of measles; 1 strain of septic sore throat, 1 strain from Rosenow's ulcerative colitis case; 1 strain from Rosenow's case of acute anterior poliomyelitis; 12 strains of green-producing streptococci from various human infections; 16 strains of anhemolytic streptococci of human origin, and 38 strains of hemolytic *pyogenes* strains. In not a single instance did "sludge" phage show the slightest suggestion of lysis. The specific lytic effect of this bacteriophage only upon the two strains of streptococcus mentioned above is very striking. It was of interest therefore to study the nature of this resistance as compared to the same phenomenon encountered in various strains of bacteria generally susceptible to the phage.

From the studies on the bacteriophage phenomenon it is evident that two types of resistance to phage can be differentiated.

1. *Apparent Resistance*.—Among the genera susceptible to phage, occasional strains can be found which do not undergo any lysis when brought into contact with a given principle. However, these strains are able to regenerate bacteriophage and carry it from generation to generation without being attacked themselves.

\* The first three points were published by Clark and Clark when this work was almost completed.

2. *True Resistance*.—Certain genera of bacteria for which no bacteriophage was as yet found; or occasional strains of susceptible bacteria; or individual colonies of bacteriophage carrying strains show complete indifference to this principle. They are not attacked by it and are not able to regenerate it. In order to determine to which of these categories the resistance of streptococcus belongs, experiments were made in a quantitative manner and the fate of "sludge" phage in resistant cultures was followed up. Strains 55 (Williams—scarlet fever); E, (Birkhaugh—erysipelas); V-(*viridans*—endocarditis); H(*hemolyticus*—meningitis) were used for these experiments. Flasks of broth containing  $1 \times 10^{-5}$  cc. dilution of Clark's phage ( $E_L-8$ ) were inoculated with these strains. In 24 hours the cultures were filtered and the filtrates tested in various dilutions against "Rb" streptococcus. All these filtrates were able to produce lysis of "Rb" streptococcus in dilutions up to  $1 \times 10^{-2}$  cc. only. It is evident that no regeneration of lytic principle was attained by any of the streptococci employed in these experiments. The conclusion was, therefore, that streptococci showed true resistance to lytic principle. In view of the fact that reproduction of the bacteriophage phenomenon is impossible without the ability of regeneration on the part of bacteria, it was thought advisable to direct the work towards creating favorable conditions which would enable streptococci to regenerate phage. Should these efforts fail it would then be of interest to attempt to train streptococci to perform this function.

## II.

### *Adaptation of Various Lytic Principles to Hemolytic Streptococci.*

In this experiment the usual method of "adaptation" of phage to a resistant microorganism was used. Various anti-*coli* phages, B.H. and "sludge" phage, were titrated against three strains of scarlet fever streptococci (Williams—55, 108, 130); one strain of erysipelas (Birkhaugh— $E_1$ ), and five strains of *pyogenes* hemolytic streptococci. In 24 hours each titration was then pooled in a flask, diluted with broth, and incubated for a few days. These cultures were filtered, the filtrates reinoculated with the respective strains, some lytic principle added, and the mixtures incubated again. This process of successive filtration,

reinoculation, and incubation was repeated four times. The final filtrates were tested against the above mentioned strains of streptococci. Occasionally, a filtrate used in dilution 1:10 would show a suggestion of lysis. However, it was never possible to duplicate such a result and all these trials remained frankly negative. Since in the majority of instances exact record was kept of the amount of lytic principle added, it was possible to determine by means of experiments described in Part I that there was never any regeneration of these principles.

*The Influence of Age of Culture upon Susceptibility to the Bacteriophage.*

The original lytic principles as well as various filtrates of the preceding experiment were tested against the above mentioned scarlet fever streptococcus cultures of various ages from 6 hours to 1 month old. Only in one case, "pig" anti-*B. coli* phage showed complete lysis with 1 month old culture of Strain 55 without regeneration however. It was impossible to repeat this result. All the other results were distinctly negative.

*The Influence of Various Degrees of Anaerobiosis Tested at Different Hydrogen Ion Concentration upon the Susceptibility of Streptococci to Bacteriophage.*

The author has previously demonstrated (7) that a quantitatively weak principle was able to produce complete lysis in culture of *B. coli* placed under partially anaerobic conditions in cases where it failed to do so under frankly aerobic conditions. Moreover (8) definite reciprocal relationship had to be observed between the hydrogen ion concentration and oxygen tension in order to enable *B. coli* to retain its susceptibility to phage. These observations were applied to the studies on streptococci with the hope that more favorable conditions would be found for obtaining lysis and regeneration of bacteriophage. For this purpose Strains 55 and 108 were cultured for three generations in broth of different pH under various oxygen tension conditions. The different cultures were then tested against several lytic principles under similar conditions. No lysis was observed in any of these cultures and no regeneration obtained.

*The Influence of Temperature on Lysis of Streptococci.*

Experiments with principle B. H. and staphylococcus showed the following: If B. H. was not regenerated for a period of 2 to 3 weeks it was apt to lose its potency almost completely. However, if a culture of staphylococcus containing such a principle was left at room temperature for a few days sudden complete clearing occurred. Such a lysed culture then contained a lytic principle which was able in turn to produce prompt lysis at 37°. It was decided to apply this observation to the studies upon streptococci and combine it with the method of the preceding experiment. The preceding experiment was repeated but cultures containing lytic principle were left at room temperature for over 2 weeks. No lysis appeared under these conditions and no regeneration of lytic principles was noticed.

*Addition of "Activating" Factors to Cultures of Resistant Streptococci.*

On the assumption that spontaneously resistant strains of streptococci may not produce regeneration on account of the absence of some activating factors, the following experiments were undertaken: These hypothetical factors were looked for in cultures of "Rb" streptococcus which always showed remarkable susceptibility. Cultures of 55,108, and 130 strains were grown in broth containing a suspension of live "Rb" streptococcus, or filtrate of live culture, or a suspension of this culture autolysed by freezing and thawing. (Autolysis was made aerobically and anaerobically.) In no instance did the strains prepared in such a manner show any change in susceptibility to "sludge" phage.

*"Training" of Streptococci to Regenerate Bacteriophage.*

The lack of power regeneration under various supposedly favorable conditions suggested the necessity of employing some method which would "train" the strains under question to perform this function. In the methods ordinarily employed the lytic principle was "adapted" to the microorganisms. In contrast to this method (page 501), the author attempted to "adapt" the microorganism to the lytic principle. Various strains of human pathogenic streptococci chosen for this experiment were subcultured every 24 hours in broth containing 1:10 dilution of lytic principle. After several such passages

the cultures were inoculated into flasks of broth, incubated for 24 hours, filtered, and 0.5 cc. of each of these filtrates tested against homologous normal cultures of streptococci. Table I represents the results obtained.

As is seen from this table, sixteen strains of hemolytic streptococci of erysipelas origin acquired the property of regenerating lytic principle active against normal cultures of their homologous strains. The

TABLE I.

Strain of streptococcus	Lytic principle	Number of passages through phage	Degree of lysis of normal culture vs. filtrate of "adapted" culture of the same strain
Birkhaugh erysipelas E <sub>1</sub> .....	C/2	15	0
10 erysipelas strains (Birkhaugh) E <sub>1</sub> -E <sub>10</sub> .....	"Sludge"	12	4+
Erysipelas—E.....	C/2	36	0
<i>Viridans</i> subacute bacterial, endocarditis.....	"Sludge"	30	0
Scarlet fever 55, 108, 130, 4, 84, 42, "L", "M".....	"Sludge"	20	0
Scarlet fever 55, 108, 130.....	"Sludge"	70	0
"Rb" streptococcus.....	C/2	15	0
"Rb" streptococcus.....	"Pig"	20	0
<i>Pyogenes</i> hemolytic 2 strains.....	"Sludge"	37	0
Scarlet fever 55, 108, 130.....	C/2	19	0
Scarlet fever 55, 130.....	"Pig"	18	0
2 strains <i>pyogenes</i> hemolytic streptococcus.....	C/2	31	0
Erysipelas strains Birkhaugh 243, W 248, 214, 268.	"Sludge"	12	4+
American Type Culture Collection erysipelas 766, 777.....	"Sludge"	12	4+
American Type Culture Collection erysipelas 768, 425.....	"Sludge"	12	0
769 Birkhaugh 239, 218.....	"Sludge"	12	0

same method failed to give any results with the same principle cultivated with other pathogenic streptococci, and another lytic principle failed to give results with the erysipelas strain. The following two explanations offered themselves to account for the failure of numerous other streptococci to acquire the property of regeneration.

It is quite possible that the strains which could be eventually trained to regenerate phage normally possessed this property in a *latent* condi-

tion before the experiment began, while the other strains which failed to acquire this property by this method—*entirely* lacked it. However, C/2 phage failed to evoke this property in a strain of erysipelas streptococcus, which was shown to undergo this change with “sludge” phage. There remained, therefore, the second possibility, namely that the phage employed must also possess a special ability to evoke such a change. The question as to whether the resistant streptococci would not eventually demonstrate the function of regeneration if only a proper phage is offered to them in the process of “training” is at present being followed up.

*Streptococcus Erysipelas Bacteriophage.*

The following observations were made on the erysipelas streptococcus bacteriophage:

Quantitative and qualitative estimation of potency of phage was made by titration in broth. The filtrate of a broth culture of a strain which was trained to regenerate a powerful lytic principle showed lytic exponent  $-4$ . The last tube showing lysis was filtered. The filtrate then showed  $E_L - 8$ . Such a lytic principle could be kept at this titer and transmitted indefinitely through contact with normal cultures of erysipelas strains. Care had to be taken to regenerate the phage frequently since the lytic principle had a tendency to partially lose its potency on standing. As is seen from these observations, the lytic principle regenerated by a “trained” strain was afterwards easily regenerated by normal cultures. Evidently, therefore, the process of obtaining phage for a resistant strain consists of the following stages:

(a) “Adaptation” of such a strain to the “sludge” lytic principle bringing about appearance of power of regeneration. (b) Change of the “sludge” lytic principle offered to the strain to a state which allows its regeneration by a normal and, therefore, unprepared culture of the same strain. To sum up the results so far obtained: (1) It is interesting to note that resistant strains of streptococcus may lack completely the ability of regeneration for one type of phage (C/2). (2) They may have a “latent” potentiality of regeneration for another type of phage (“sludge”). (3) They may have this property in an active state for the third type of phage (erysipelas phage).

The strain of erysipelas streptococcus which was "trained" to regenerate an antierysipelas streptococcus phage was studied.

1. When a subculture on solid medium was tested against streptococcus erysipelas phage it showed complete resistance as demonstrated by the absence of "eaten up" areas in those areas where a drop of phage was put on the surface of a bacterial film. Nor did any suggestion of lysis occur in fluid medium.

TABLE II.

	"Sludge" phage vs. Rb streptococcus	"Sludge" phage vs. erysipelas streptococcus	"Erysipelas" phage vs. Rb streptococcus	"Erysipelas" phage vs. erysipelas streptococcus
Degree of lysis.....	4+	0	4+	4+
Lytic exponent.....	-8	0	-8	-8

TABLE III.

*Degree of Lysis.*

Dilutions of sera	-1	-2	-3	-4
Serum 87 vs. 0.5 cc. "sludge" phage + Rb streptococcus..	0	0	0	0
Serum 87 vs. 0.5 cc. erysipelas phage + erysipelas streptococcus.....	0	0	0	0
Serum 212 vs. 0.5 cc. of "sludge" phage + Rb streptococcus.....	0	0	4+	4+
Serum 212 vs. 0.5 cc. erysipelas phage + erysipelas streptococcus.....	0	0	0	0

4+ = complete lysis; 0 = normal growth.

Serum 87 prepared by immunization with "sludge" phage.

Serum 212 prepared by immunization with erysipelas phage.

2. The above mentioned strain was also streaked on the surface of a plain agar plate. Microscopic examination of colonies appearing in the course of 1 week did not show any striking morphological changes such as described for other microorganisms acted upon by phage.

The following colonies were observed: large smooth but slightly irregular with occasional erosions in the center or at the margin of the

colony; small smooth colonies with irregular margin; irregular fine masses of cocci without any definite colony formation. These colonies were grown in broth and tested against erysipelas bacteriophage. They all showed the same degree of resistance. The filtrates of fluid cultures contained active lytic principle. It was, therefore, impossible to obtain resistant but bacteriophage-free colonies, such as can be done with other microorganisms. A more painstaking search should, however, be made. The above observations leave no doubt that the lytic principle obtained is in no respect different from the classical bacteriophage.

TABLE IV.

Strains tested vs. erysipelas phage	Lytic exponent	Degree of lysis
10 strains of streptococcus erysipelas (Birkhaugh) E <sub>1</sub> -E <sub>10</sub> .....	-8	4+
Birkhaugh, erysipelas streptococcus 243, W 248, 214, 268.....	-8	4+
American Type Culture Collection 766, 767 streptococcus erysipelas.	-8	4+
Williams, scarlet fever streptococcus 55, 130, 108, 4, 84, 42.....	0	0
Rb <i>Streptococcus hemolyticus</i> .....	-8	4+
Mt. Sinai, scarlet fever streptococcus "M", "L".....	0	0
Streptococcus erysipelas, Birkhaugh 239, 218.....	0	0
Mt. Sinai, hemolytic pyogenic streptococci, 36 strains.....	0	0
American Type Culture Collection streptococcus erysipelas, 768, 425, 769.....	0	0
Mt. Sinai, green-producing streptococci, various conditions, 8 strains.....	0	0
Mt. Sinai, anhemolytic streptococci, 5 strains.....	0	0
American Type Culture Collection anhemolytic streptococci 421, 422, 423, 349, 345 hemolytic, sore throat 543.....	0	0

In order to determine the relations of "sludge" phage to the erysipelas lytic principle, antiphage sera were prepared with both phages. Care was taken to "purify" the erysipelas phage by several transmissions through normal cultures of erysipelas strains. Tables II and III represent the results obtained.

As is to be noted, both phages are able to lyse "Rb" streptococcus, while only erysipelas phage is able to dissolve the erysipelas strain. It can be assumed, therefore, that both phages contain a common component and that the erysipelas phage has also a specific portion which makes possible lysis of erysipelas streptococcus. Since the "sludge" phage serum is also able to neutralize erysipelas phage it is apparent



that the presence of the common component in addition to the specific portion is essential for the action of the erysipelas phage. A difference can be detected in the effect of antierysipelas phage serum upon both phages. This serum neutralizes the "sludge" phage in dilution 1:100 and the erysipelas phage in dilution 1:10,000. No explanation is at present offered for this result.

Studies on the specificity of the erysipelas streptococcus bacteriophage are recorded in Table IV.

Thus this bacteriophage is able to attack about 76 per cent (16 out of 21) of all the tested hemolytic streptococci of erysipelas origin besides the "Rb" streptococcus. The specificity of this phage is of extreme interest. As is seen, no other streptococci out of 64 strains tested is attacked to the slightest degree. It would be of interest to study the relation of the specific agglutinogen of this group to the susceptibility to this phage.

#### SUMMARY AND CONCLUSIONS.

1. The "sludge" phage obtained by Clark and Clark answers all requirements for pronouncing it identical with the classical bacteriophage.

2. The "sludge" phage failed to produce lysis in any of the 102 human pathogenic streptococci tested.

3. Numerous attempts to induce regeneration of various lytic principles by human streptococci resulted in failure.

4. It was possible, however, to "train" erysipelas streptococci to regenerate a lytic principle active against 76 per cent of strains of this group.

5. The erysipelas phage showed remarkable specificity.

I wish to express my thanks to Miss G. Ethelbert McKennon for her assistance.

#### BIBLIOGRAPHY.

1. Piorkowski, G., Beitrag zur Streptokokkenfrage. Anwendung des d'Héréleschen Phänomens auf Streptokokken, *Med. Klin.*, 1922, xviii, 474.
2. McKinley, E. B., Notes on d'Héréle's phenomenon. Adaptation of bacteriophage antagonistic to *Bacillus dysenteriae* and other bacilli to various cocci. Development of a polyvalent bacteriolysant, *J. Lab. and Clin. Med.*, 1922-23, viii, 311.

3. Dutton, L. O., Rôle of bacteriophage in streptococcus infections; an interpretation of certain cultural characteristics, *J. Infect. Dis.*, 1926, **xxxix**, 48.
4. Hadley, P., and Dabney, E., The bacteriophagic relationships between *B. coli*, *S. fecalis* and *S. lacticus*, *Proc. Soc. Exp. Biol. and Med.*, 1926, **xxiv**, 13.
5. Clark, P. F., and Clark, A. S., A "bacteriophage" active against a hemolytic streptococcus, *J. Bact.*, 1926, **xi**, 89.
6. Clark, P. F., and Clark, A. S., Bacteriophage against virulent streptococcus, *Proc. Soc. Exp. Biol. and Med.*, 1927, **xxiv**, 635.
7. Schwartzman, G., Studies on regeneration of bacteriophage. I. The influence of partial anaerobiosis upon regeneration of a highly diluted lytic principle, *J. Exp. Med.*, 1925, **xlii**, 507.
8. Schwartzman, G., Studies on regeneration of bacteriophage. II. The influence of oxygen upon the behavior of *Bacillus coli* towards lytic principle, *J. Exp. Med.*, 1926, **xliii**, 743.



# THE RHYTHMIC RANGE OF THE WHITE BLOOD CELLS IN HUMAN, PATHOLOGICAL LEUCOPENIC AND LEUCOCYTIC STATES, WITH A STUDY OF THIRTY-TWO HUMAN BONE MARROWS.

BY CHARLES A. DOAN, M.D., AND LEON G. ZERFAS, M.D.

*(From the Thorndike Memorial Laboratory, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.)*

(Received for publication, June 15, 1927.)

In an attempted interpretation of the occasional unexplained inconsistencies in clinical blood counting it has been suggested (1) that there is normally a more or less rhythmic variation in the numbers of white blood cells from moment to moment in each individual. In general the total number of white blood cells has been found to vary, within physiological limits and without abnormal external or internal stimuli, 100 per cent during the course of a day in a single, apparently normal, adult individual. In other words, rather than a relatively fixed individual "norm" or concentration of circulating cells, our concept of the normal for each person now is a wide zonal range, with a fluctuation for example of from 4000 to 8000 or from 6000 to 12,000 white blood cells, any chance single count being the resultant of at least three variables, the person, the physiological conditions of the moment, and the time of day of the observation. A. F. Bernard Shaw (2) has recently confirmed this concept and has carried his observations further by describing diurnal tides of the leucocytes in man, finding consistently two low points during the 24 hours, one between 9 and 11 in the morning, the other between midnight and 2 a.m., entirely independent of starvation, food, rest, exercise, or sleep. Bushnell and Bangs (3) in surveying the large individual variations in both white and red cells occurring in normal rabbits, conclude from their mean of 10,675 that  $\pm 4800.75$  must be taken as the limits of normal for determinations of the white cells, and from a mean of 5,989,500,  $\pm 1,682,100$  for the range of normal for the red cells. In one normal

animal daily and hourly counts gave as extreme limits for the white cells 10,200 and 13,600, for the red cells 4,322,000 and 6,976,000. For a review of the literature and an analysis of the normal rhythmic variations of the white cells the reader is referred to the three articles above mentioned.

The question which arises immediately in the mind of the clinician, habituated to depend more or less upon the leucocyte count in disease, is as to the dependence to be placed in single counts in the light of the demonstrated 100 per cent, normal individual fluctuation. To the end that we might possibly determine something concerning the mechanism of, and range for, the white blood cells in disease the series of studies here presented were undertaken. We have been guided in our experimental observations by an attempt to answer the following questions: Are the observed variations in total numbers of white blood cells in the same individual from time to time fancied or real? How large a factor is the error inherent in the technic of counting? Can rapid changes in blood volume account for the variable counts? How often and how regularly do the changes come? In short, is there an analyzable inherent rhythm to the fluctuation in the total white blood cells in disease, and do the different types of white cells maintain independent rhythms? If so; do they parallel the established normal variants, *i.e.* is a leucopenia simply the manifestation of a depression, a leucocytosis the result of an accentuation, of the normal mechanism? Is it important to do more than one white count for an accurate working knowledge of the available cells at any one period in the course of a disease, and, if so, how many and at what intervals should the repeated observations be made? Can we arrive at any idea of the rate and manner of delivery of cells from the bone marrow by an analysis of consecutive observations on the peripheral blood at 15 minute intervals? In summary, are the apparent fluctuations in total numbers of white cells actual changes in the absolute numbers of potentially available circulating units, or do such changes represent technical, observational inaccuracies plus a vasomotor, redistribution phenomenon with percentage importance only? Entirely irrespective, however, of the desire to understand and to explain the mechanism in these observations, is the fact that controlled consecutive counts do register large variations which must

be taken into consideration when the leucocyte count may be an important factor in diagnosis or prognosis and treatment in disease.

We have studied a wide series of clinical conditions in which both leucopenia and leucocytosis of varying degree have existed. The twenty selected cases presented in this report are but representative of similar findings in a much larger survey of cases, but in which the data are less complete. As a routine each patient has been followed for a period of 3 hours, at intervals of 15 minutes, certain cases having been observed on successive occasions for similar unit periods. This permits of the construction of a graphic record with a more accurate interpretation of the limits and meaning of the variations. An automatic lancet was used, thus theoretically insuring consecutive samples of freely flowing blood from the same depth each time. A different finger was used for each observation, or a different portion when it was necessary to use the same finger again toward the end of a 3 hour experiment. The order of procedure was as follows: first drop discarded, supravital preparation, cover-slip spreads for Wright's stain, total white blood cell count, total red blood cell count, hemoglobin, hematocrit, serum protein. In the complete studies two of us were making the preparations, each taking the same type of sample each time, so that usually within 2 minutes all tests had been secured and the individual personal error in each particular series should have been fairly constant. In several of the cases a double check of the total white blood cell count was made by two persons independently to determine the error inherent in this technic. The same Bureau of Standards equipment was used throughout in each series of observations. The hemoglobins were read in a Duboscq colorimeter with the Newcomer standard. The hematocrit determinations were made with the Van Allen pipette (4). Refractometric readings were made for serum protein determinations. Our problem has not been concerned so directly with the establishment of exact absolute values for hemoglobin, serum protein, and hematocrit readings as with the relationship existing between consecutive *ad seriatum* determinations made under identical, standardized, closely controlled conditions and bearing to each other a definite time relationship. This might be expected to give at least the relative variations in a given determination over the period of observation. In some instances a 100 cell supravital differential count was made, with vital neutral red (5, 6); in others cover-slip preparations were made with Wright's stain and 200 cells counted by two observers from both cover-slips and the average percentages taken; in some cases both supravital and fixed film observations were made and compared. We have found that while it is essential to count larger numbers of cells and average the counts from both cover-slips to obtain accurate data from fixed films, with the supravital preparations, in which the whole drop of blood is evenly and uniformly spread out, 100 white cells counted will give in the majority of instances the same percentage incidence of cell types as a larger number of cells counted or as is given by a count of two consecutive preparations by two different individuals. In certain of the cases we have made an

Arneth differential count uniting for charting the non-lobulated and two lobed nuclei as significant of the younger mature polymorphonuclear neutrophils, the three and four lobed nuclei as fully mature, and, those having five or more lobes as indicating old cells. In the supravital preparation we have differentiated the actively motile leucocyte from the round inactive form and from the specific "non-motile" neutrophil of Sabin (5), the last being interpreted as the morphological state denoting the physiological death of the cell.

In addition to the series of twenty cases, which form the basis of the clinical observations herein analyzed, differential cell counts of the bone marrow have been

TABLE I.

Case	Age	Sex	Diagnosis	W.B.C.	Range 3 hr. period with 15 min. counts
	<i>yrs.</i>				
1 (T71)	35	M.	Chr. osteomyelitis	Leucopenia	1,400- 2,800
2 (T44)	40	"	Primary anemia	"	2,900- 5,300
3 (T5)	65	"	" "	Low normal	4,000- 6,500
4 (T86)	25	"	Ac. rheumatic fever	Normal	4,200- 7,000
5 (T87)	22	"	" " "	"	4,200- 8,000
6 (T89)	25	F.	" " "	"	4,400- 10,200
7 (T111)	30	"	Erythrodermia	Leucocytosis	7,400- 15,100
8 (T81)	75	M.	Chr. cystitis	"	9,500- 15,500
9 (T88)	24	F.	Ac. rheumatic fever	"	8,800- 16,500
10 (T90)	18	"	Chr. otitis media	"	10,800- 16,500
11 (T41)	40	M.	Lobar pneumonia	"	10,000- 20,000
12 (T4)	55	"	Polycythemia vera	"	10,500- 23,000
13 (T92)	40	"	Amebic dysentery	"	14,700- 24,600
14 (T78)	16	F.	Pertussis	"	17,000- 25,000
15 (T82)	15	M.	Rheumatic endocarditis	"	16,000- 32,000
16 (T109)	30	"	Lung abscess	"	23,000- 34,600
17 (T110)	40	"	Septicemia (strep.)	"	23,900- 34,700
18 (T63)	30	"	Lobar pneumonia	"	50,000- 75,000
19 (T70)	60	"	Chr. lymph. leucemia cutis	"	35,000- 52,000
20 (T112)	45	"	" myeloid leucemia	"	208,000-284,000

made from thirty-two selected cases coming to autopsy in the Pathological Department of the Boston City Hospital. This has given additional independent evidence in confirmation of the probable part played by the bone marrow in white blood cell fluctuations, more spectacularly in the leucocytoses. These latter preparations were made originally (L.G.Z.) with an entirely different end in view and only on subsequent analysis was it seen how strikingly an interpretation of these data corroborates the rhythmic concept developed from a study of the peripheral white cell fluctuations in health and disease. In the postmortems performed within 5 hours after death, supravital differential studies were made to

check with the fixed films (7). However, in the majority of cases the counts were taken from fixed preparations. In all instances, except as specifically indicated, the femoral bone marrow was used. Occasionally it was convenient to examine

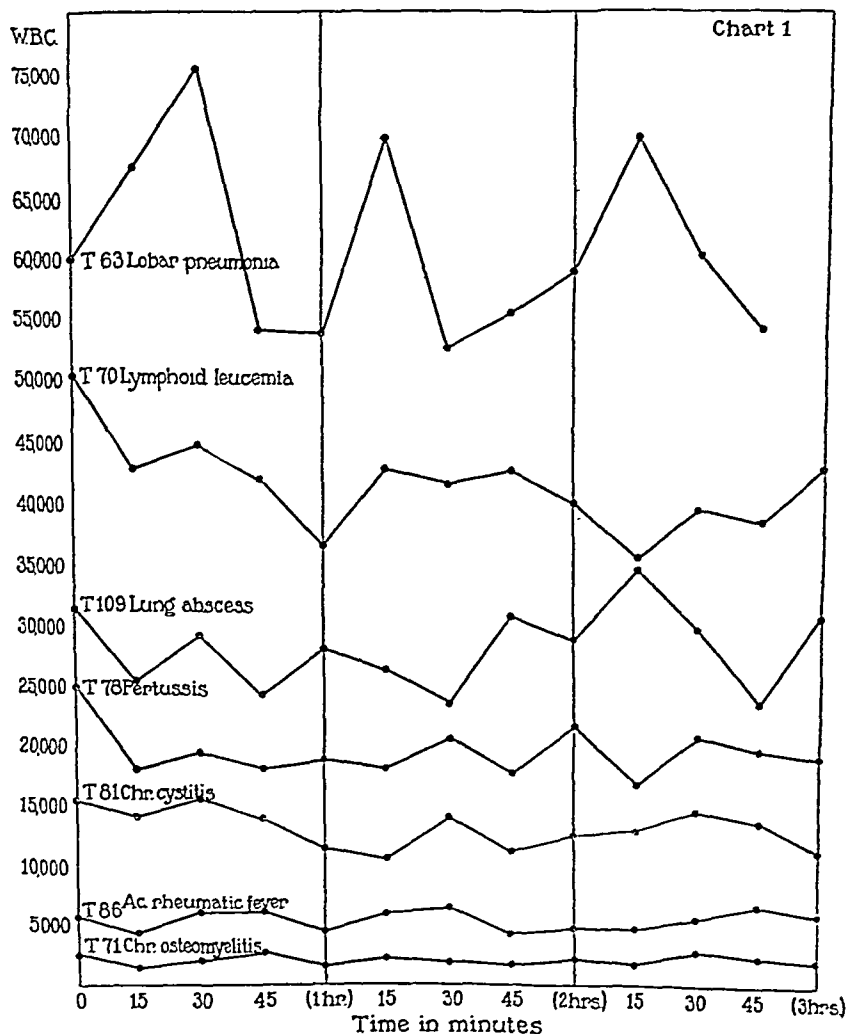


CHART 1. Fluctuations in total white cell counts.

vertebral marrow. In the empyema cases, the marrow from ribs removed at operation formed the basis for the counts. All fixed preparations were made as follows: A small piece of bone marrow was emulsified in 2½ per cent sodium citrate, and centrifuged at high speed (2600 R.P.M.) for 10 minutes. The supernatant



fluid with the fat was pipetted off and the cells were rewashed three times with sodium citrate, the suspension being centrifugalized each time. The washed cells were then suspended in a few drops of autogenous serum, from which coverslip preparations were made. After drying in the air, they were stained with Wright's stain and mounted in balsam. No fewer than 1000 cells were counted in any case and as many as 2000 cells were counted in some instances, by two observers, and the average percentage recorded.

In Table I will be found the clinical cases on which at least one 3 hour period of observation was made. The patients' age, sex, diagnosis, and range of total white blood cells during the 3 hour period are given. In Chart 1 are to be seen graphic representations of the white blood cells in selected cases showing the variations in the total white cells at different levels—from a leucopenia of from 1400 to 2800 cells to a leucocytosis of from 50,000 to 75,000.

#### *Controls in the Establishment of the White Cell Variations.*

In the attempt to establish in so far as might be possible the nature of the apparent fluctuations in the total white cell count, coincident and simultaneous observations of the total red blood cells, hemoglobin, hematocrit, and serum protein were made, all or in part, in five cases, as recorded in Charts 3A, 8A, 10A, 11A, and 12A. The curves of the red cells, hemoglobin, and hematocrit tend to follow each the other but with no relationship to the total white cell curve. The serum protein produces the most constant finding, the very slight changes noted in Chart 12A bearing no relationship to the fluctuations in the other curves, and in Chart 8A there was a constant reading throughout the whole period except for one slight rise. It has been generally accepted that the serum protein (8), under normal basic, fasting conditions is one of the most constant constituents of the blood, relatively, and by pediatricians, in particular, has been used extensively as an index of dehydration. This fact, together with the ease of refractometrically determining its per cent on small samples of blood caused us to decide to make these consecutive determinations.

Table II summarizes the range of total red blood cells and hemoglobin observed in selected cases in this series. Roughly, the hemoglobins run in percentage variation about half that of the cells, *i.e.*, 1 per cent variation in hemoglobin is the equivalent of a 2 per cent

variation in red cells. The tendency toward a relatively large variation between the minimum and the maximum count when consecutive observations on the same individual are compared, whether the time interval be in minutes or days, has been so constant in the experience of the last several years that, for the red cells too, we believe there is a normal, individual zonal fluctuation rather than an exact point in total count which it is necessary to consider. Thus when a single count, particularly in the female patient, reveals the total red blood cells near 4,000,000 it is necessary to know whether that figure represents a minimum, the average, or a maximum point in the physiological range for that individual. As is not infrequently the case,

TABLE II.

*Red Blood Cells.**Range during 3 Hour Period with Counts at 15 Minute Intervals.*

Case No	Minimum count	Maximum count	Hgb range	R.B.C. var.	Hgb. var.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
3 (T5)	3,616,000	4,808,000	74- 85	24	13
10 (T90)	4,112,000	5,232,000	90- 96	21	6
12 (T4)	5,760,000	6,470,000	125-130	10.9	4
13 (T92)	3,696,000	4,624,000	64- 72	20	11
14 (T78)	4,880,000	5,632,000	83- 89	13	7
Normal individual (R44)	5,230,000	6,180,000	79- 88	15	10

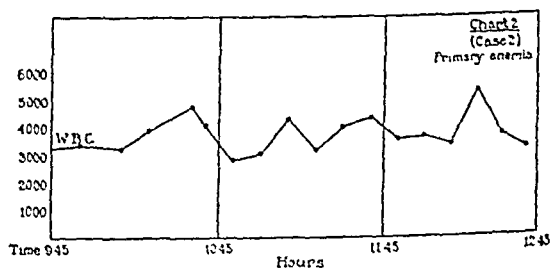
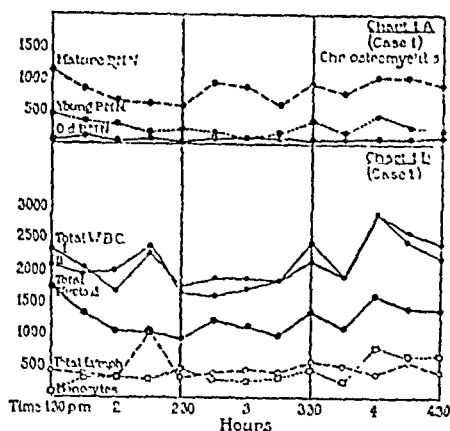
it may well be only the lower limit of a normal swing in the count for that patient if subsequent counts register 4,500,000 to 5,000,000. Only a consistent trend upward or downward in both hemoglobin and total cells as determined in repeated observations on the same individual over a period of days may provide a reliable basis for an estimate of the state of equilibrium, or the lack of it, in the supply of red cells from the hemopoietic organs. The lack of correlation between the curves of the serum protein, the white and the red cells in the present series of cases minimizes the factors of blood volume and circulatory disturbance as solely responsible for the observed variations. Rather the now well recognized reservoir for red cells in the spleen (9) and the known periodic delivery of new cells from the bone marrow (as re-

vealed, for example, in "normoblastic crises") must be considered as potential factors of importance.

### *Leucopenia.*

The lowest maintained white blood cell count which was observed during the year was that of Case 1 (Charts 1A and 1B) in the series.

The diagnosis of chronic osteomyelitis of the right tibia was made. Repeated white blood cell counts made singly on successive days during the patient's course in the Hospital consistently registered below 4000. During the 3 hour period of the consecutive 15 minute observations the lowest number of white cells counted



was 1350, the highest 2850. In this instance two of us took white blood cell counts simultaneously and counted them independently in an endeavor to check the error inherent in the technic of counting (see also Charts 7B, 17A). Because of the marked leucopenia blood was drawn to the 1 mark in the pipettes instead of the usual 0.5. The greatest single variation from the two pipettes was 700 cells; the greatest variation in the 3 hours, 1500 cells. Thus the maximum error attributable to technic in this particular case is 47 per cent. However, the majority of the counts checked within a narrower range and the technical error in general where the total number of cells is larger may be depended upon not to exceed 10 per cent. The actual cell differences in the two counts each time are as follows:

400	250	500
690	650	0
700	250	25
160	100	225
		525

Obviously the smaller the number of cells the greater the percentage of error in the counting on the basis of the computations necessary in the present method of estimating total cells per c.mm.

At such points as the counts taken at 2.00 and at 2.45, where instead of the usual paralleling of the total cell change in the two pipettes the two counts diverge slightly, the average of the two, of course, gives a concept no nearer the true count than either of the extremes. Consequently such results admit of no analysis or interpretation. It is only in counts such as those at 3.45 and 4.00 where the changes are relatively marked and registered in both observations that one feels justified in trying to explain the variation on some basis other than technical. The practical importance of this point is, of course, only apparent in later cases with higher counts.

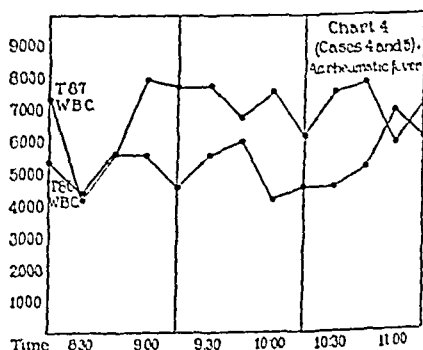
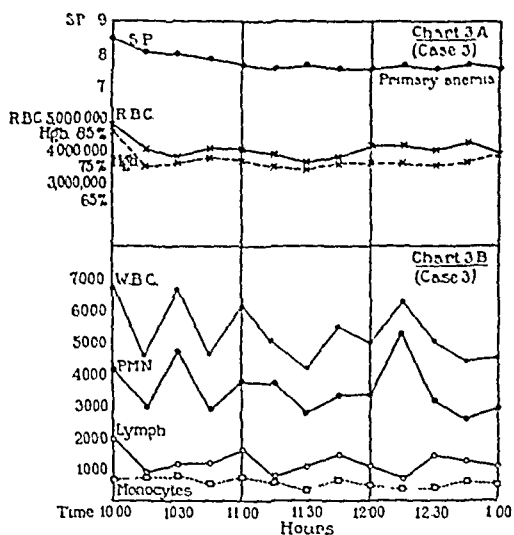
On Chart 1B are outlined the absolute numbers of myeloid, lymphoid, and monocytic cells, based on differential white blood cell counts on fixed preparations stained with Wright's stain, and the average total white cell counts. It will be noted at once that these three types of the white cells vary independently of each other. The increase of lymphocytes at 2.15 is responsible for the peak in the total white cell curve at that moment. The two elevations at 3.30 and 4.00 follow increases in the absolute numbers of myeloid cells, and for the 3 hour period there are four elevations roughly speaking for this group. The monocytes in this case illustrate the tendency to a 45 minute or hourly rhythm which we have noted in a number of cases. Monocytes also vary, rising and falling over periods of several days, as in typhoid fever and tuberculosis. This independent variation in the percentage and total numbers of the three types of white blood cells is the first evidence against a simple dilution explanation for the variations in the total cell count. It will be seen later and more strikingly in other cases. This case may illustrate also the fact that there is a tendency, as found in the normal, to a higher late afternoon white count since the only two counts over 2500 came after 4.00 p.m. The relative proportions of the three types of cells are approximately normal, *i.e.* there is not the relative lymphocytosis frequently seen in leucopenia. However, in so low a total count as is this, it is reasonable to expect the lymphocytes to influence more readily the total count at times. Thus the two groups of cells must be taken into account as mutually potentially influential on the total count in the leucopenic state.

Shaw (2) found, in single determinations in 116 healthy male adults, counted uniformly between 9 and 10 a.m., a percentage of lymphocytes equal to or exceeding the percentage of neutrophils in 10 persons, though subsequent observations in the same individuals usually gave the general excess of neutrophils. An interesting confirmation of the transitory nature of this lymphocyte preponderance was revealed in two of his studies on the diurnal tides, in which the percentage of lymphocytes temporarily exceeded the percentage of neutrophils for some hours. While the lymphocyte curves are not given in the paper, his analysis of the differential counts showed the fluctuations to be largely in the neutrophil group, so that it may be assumed that the transitory lymphocytoses were relative, due

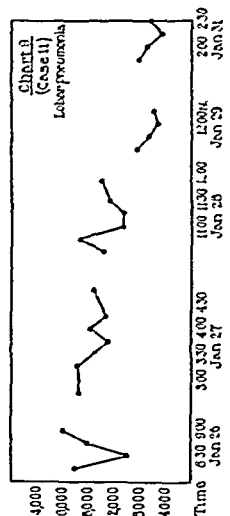
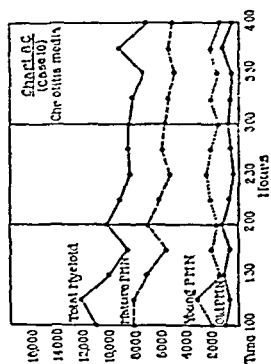
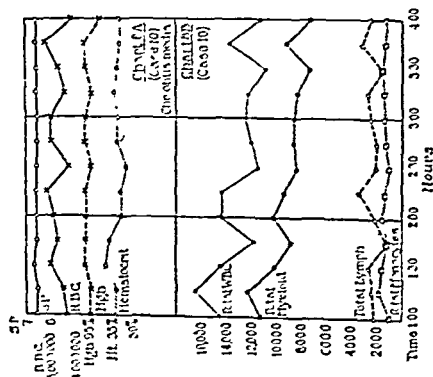
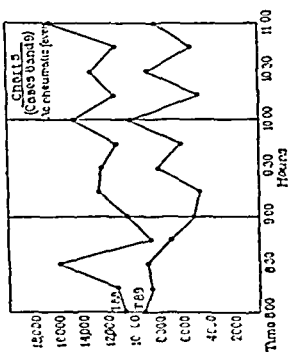
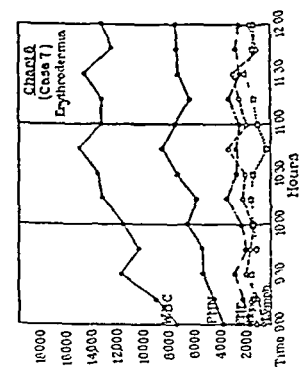
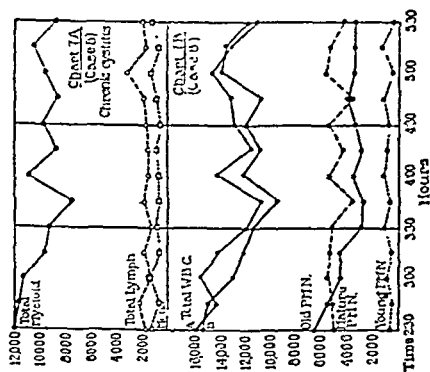
rather to the decrease of granulocytes than to any marked increase in absolute numbers of lymphocytes (10).

Chart 2 (Case 2) is representative of a leucopenic range of from 2900 to 5300 white blood cells in a 3 hour period of observation on a case with the diagnosis of pernicious anemia. In this particular instance the counts were taken at 10 minute intervals to see if any marked difference in the nature and range of the fluctuations could be discerned. Four elevations alternate with lower counts between, two peaks coming within the 2nd hour. This is not an infrequent observation, even though the usual variations at 15 minute intervals over a long period show but one rise on the average each hour.

The second case of pernicious anemia with a relatively low white count showed a fluctuation of from 4000 to 6500 white cells during the 3 hours. Charts 3A and



3B graphically depict the relationship which was found to exist between the total white blood cells, red blood cells, hemoglobin, and serum protein. Two independent series of total white counts were made again for a check on the error of counting, though not charted. It will be seen at once that the white blood cells fluctuated much more widely than, and entirely independently of, the red blood cells, the hemoglobin determinations closely paralleling the latter. The serum protein dropped gradually during the 1st hour, thereafter showing only very slight variations, between 7.55 and 7.67 per cent. On first glance it would appear that the differences in all determinations between the first and second observations, 10.00 and 10.15 a.m., could be accounted for through blood volume increase with dilution and resultant lowering of serum protein, white and red blood cells, and hemoglobin. However, the percentage change when all are reduced to the same common denominator shows the white blood cell change to be 30 per cent, red blood cell 20 per cent, hemoglobin 12 per cent, and serum protein 8.2 per cent.



Nevertheless, should such parallel changes in the same direction always occur in all the fluctuations, it would be justified to attribute small discrepancies in actual identical percentages to the error indisputably a part of each technical procedure. But this has never been found to be consistently the case. As in the instance in point, Chart 3A, the serum protein falls directly to a relatively fixed level and may be correlated with the fact that the patient was not on a fasting base line.

Chart 4 represents two series of white counts which might well serve as an example of the range of variation in individuals with a low normal total count, yet both patients had acute rheumatic fever, not yet treated, Case 4 having two active joints at the time of the count. Three elevations in the total counts may be noted in each series, during the 3 hour period. Chart 5 carries two acute cases under treatment, with the same diagnosis but with the counts in Case 9 definitely above the normal range, and each showing in the first 2 hours two peaks but in the 3rd hour two half-hourly peaks. With the recognition of the physiological fluctuations in the white cells, the influence of treatment (11), but the possible presence of activity without elevation in the total count, prognostic deductions based on small variations in the total white count in acute rheumatic fever should be conservative.

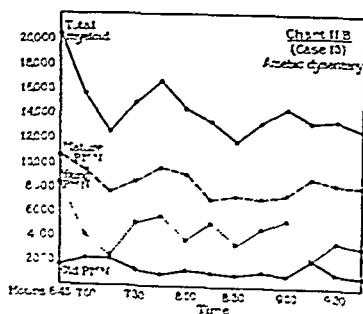
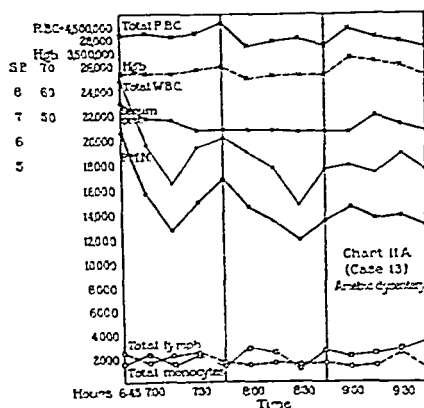
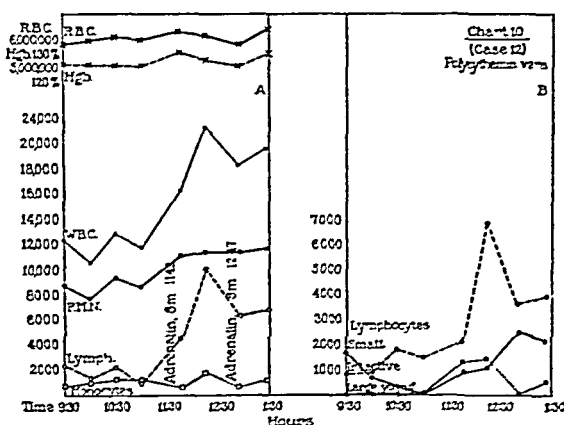
### *Leucocytoses.*

*Chronic.*—It is of interest to note the differences appearing in the chronic and acute leucocytoses. The chronic state is represented by Cases 8 and 10 as graphed in Charts 7A and B, and 8A, B, and C.

The case, No. 8, of chronic cystitis was running a white count between 9500 and 15,500, the chronic otitis media, Case 10, 10,800 to 16,500. There is a definite hourly rhythm in both cases, and the low points reached in each had they been single observations would have been equivocal in so far as a leucocytosis is concerned, particularly since they were chronic conditions and the adjustment of the bone marrow had been made on the new level. Charts 7B and 8C indicate the Arneth differentials which show a low level of young and old white cells, with the majority mature forms, even as is the case in the normal; in contrast, in the acute leucocytoses, the Arneth "shift to the left" is constant. Lymphocytes and monocytes are within their normal limits, the neutrophil group being entirely responsible for the elevation in total white count.

It will be seen strikingly again and again that particular uncomplicated pathologies stimulate independently one or another of the cell types and that the response of the organism is either limited entirely to this specific pathognomonic reaction or so predominantly to the one that the slight relative increase in the other types is obviously not a part of the specific response. Hickling (12) has pointed this out

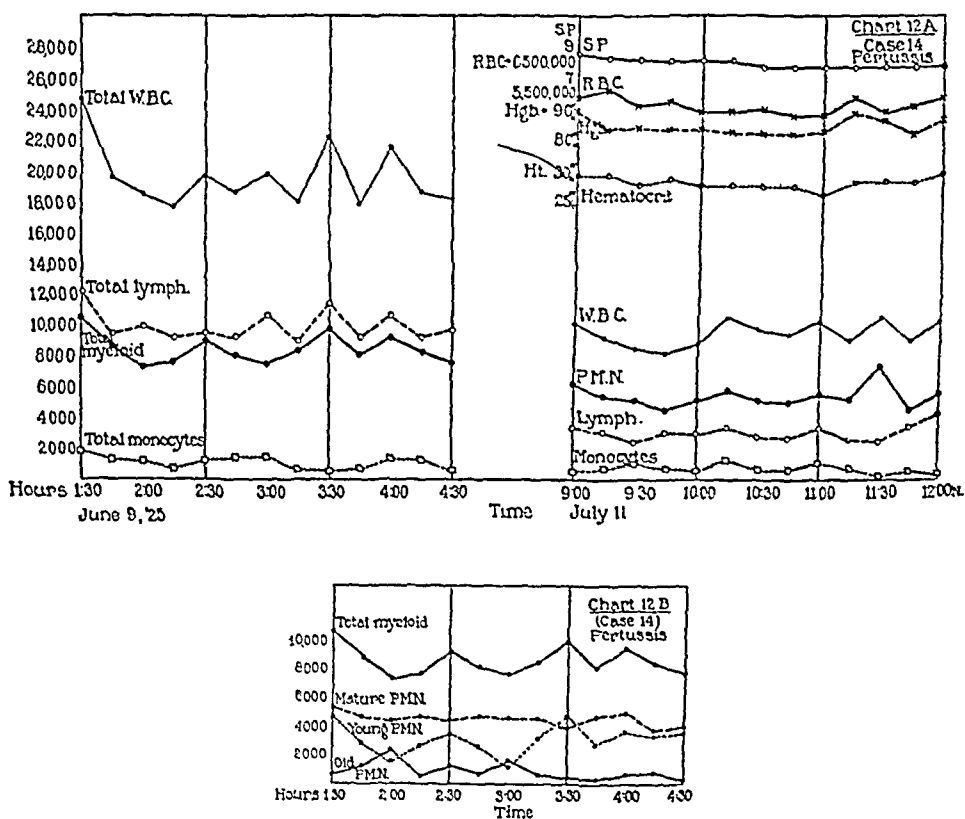
in a clinical and experimental study of toxic and chemical stimuli on the circulating leucocytes. He found a specific neutrophilic response to the presence of bacterial infection with the degree of postinfective lymphocytosis dependent upon the extent of tissue injury. In the



particular response as analyzed in the present series, the specific cell type maintains its original rhythm as will be seen in Chart 10A with lymphocytes elevated after adrenalin, in Chart 12A in which the lymphocytes are elevated in a case of pertussis, in Chart 6 in which there is an eosinophilia with erythrodermia, and in Chart 16A giving a neutrophilia typical of lobar pneumonia.



*Acute Leucocytoses.*—The group of acute leucocytoses secondary to infection are represented in six cases, two with diagnoses of lobar pneumonia, one with amebic dysentery, one with rheumatic endocarditis, one with lung abscess, and the last with streptococcus septicemia. All show the specific polymorphonuclear neutrophil increase with the "shift to the left" in the Arneth differential pattern, the younger neutrophils both showing a higher percentage than is found

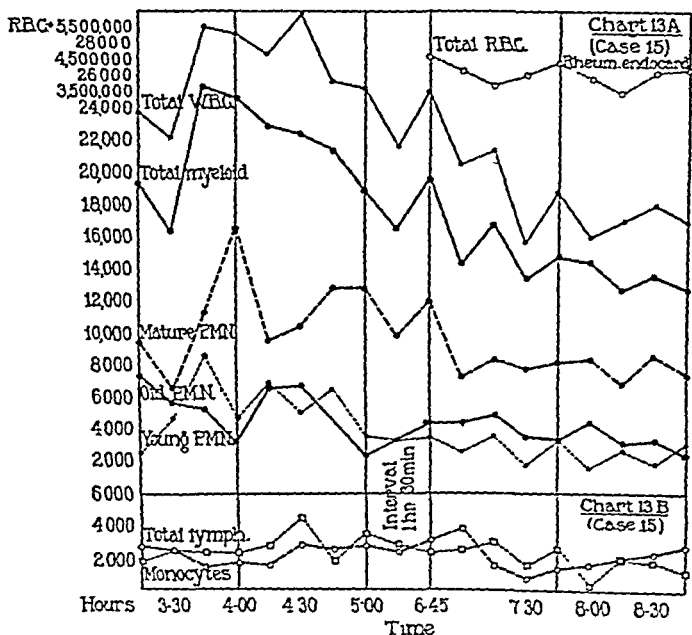


normally and the high points in the consecutive counts being seen to be a reflection of periodic increases in these forms.

Charts 11B, 14, and 15 indicate this particularly clearly. Chart 13A which combines an afternoon period with a later evening period of observation on the same day indicates the high late afternoon total count with a marked "shift to the left" of the Arneth differential and an evening count decidedly lower reflecting a lowered percentage of young cells. The lymphocytes and monocytes maintain essentially undisturbed total numbers throughout both series of counts, and the

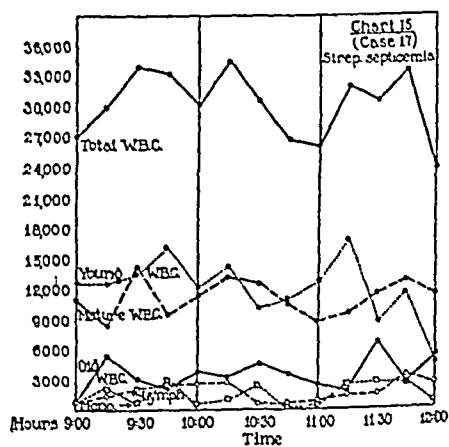
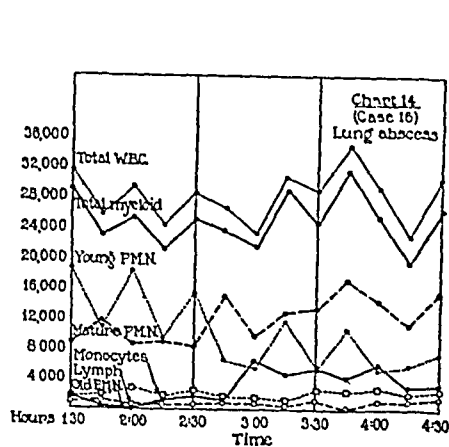
red cells during the later period show no consistent downward trend as do the white cells.

Chart 9 graphically presents the total counts done on consecutive days in a case first seen on the evening of the 3rd day of the disease. The patient gave a history of sudden onset of symptoms typical of lobar pneumonia occurring at 9.30 a.m. January 24. He arrived at the Hospital on the 26th without definite physical signs in the chest, with a temperature of 102.5°, pulse 100, and respirations 35. The white count on admission was 8000. It happened to be a period when influenza, rather than lobar pneumonia, was prevalent, so that the low total white count and physical findings in spite of the history seemed to coincide better with

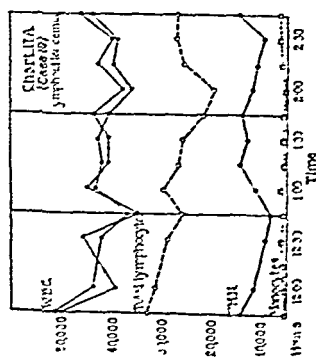
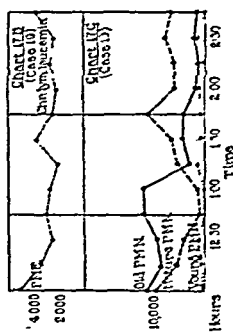
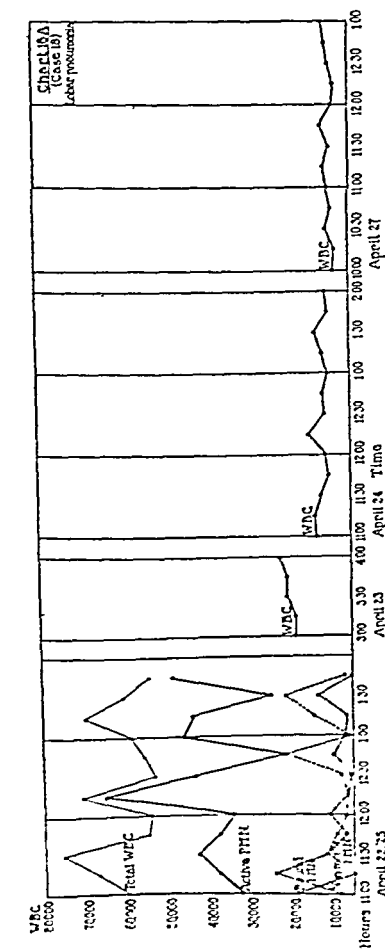


the former diagnosis. Within an hour the four counts taken at 15 minute intervals and recorded on Chart 9 had been taken. It will be seen that one of the four counts was 10,000 but that the other three were all above 16,000, one going to 20,000. Both the 8000 and the 10,000 total white blood cell counts were in all probability accurate determinations of the numbers of these cells in the peripheral blood at the moment those samples were taken, but represented only the lowest swings of an arc, which was periodically at least, indicative of a definite pyogenic stimulation. In this case there was never the marked leucocytosis seen in Case 18, but a lobar consolidation developed and a typical crisis came on the 5th day with the fall to normal in the total white count as shown under date of January 29 on the chart.

Case 18 (Chart 16A) presented the typical history and picture of a severe, uncomplicated lobar pneumonia, with complete consolidation of the right lung, temperature, at 1.00 p.m. April 22, the day of the first count,  $106^{\circ}$ , pulse 150, respirations 50. The crisis came during this afternoon, the 4.00 p.m. temperature being  $100^{\circ}$  and the 8.00 p.m. temperature  $98^{\circ}$ . The first 3 hour count, therefore, represents the maximum mobilization of the cellular defense in this patient just prior to, and almost coincident with, his crisis. It will be seen that there were three high points, one each hour, at which the total count reached or surpassed 70,000 per c.mm., but that seven of the twelve counts were between 50,000 and 60,000. The supravital differential counts revealed a high percentage of actively motile neutrophils, particularly at the moments of the highest counts, with the round and "non-motile" forms, indicating injury and cell death, relatively few in number, except at the periods of lowest ebb in the count. This is in correlation with the periodic "non-motile showers" (1) found in the counts on normal in-



dividuals. The same prognostic significance we believe is probably attached to this observation as Pons and Ward (13) attribute to the leucocyte fragility test in pneumonia, *i.e.* the larger the percentage of living cells the better the prognosis. Mauriac and Moureau (14) also analyze the curves of fragile cells most instructively. Chart 16B shows peaks in the young and mature neutrophils (Arneth differential on fixed films) corresponding with the peaks of total cells and with the high points in numbers of active cells in the supravital preparations. Chart 16C shows a normal number of lymphocytes, the pneumonic process having had no influence on this strain of cells; the monocytes show fluctuations from a normal level to a considerable increase over the usual percentage of these cells in normal blood (15). Chart 16A includes the counts taken on successive days following the crisis, showing totals ranging from 18,000 to 22,000 during only five observations on the 23rd, 10,000 to 14,000 on the 24th, and on the 27th, 6000 to 12,700. The last two periods, as the first, show the tendency toward an identical hourly fluctuation in the total cells. Whereas the polymorphonuclear neutrophils during



the period of the crisis were averaging from 89 to 98 per cent of the total count, on the 2nd and 3rd days thereafter they were 77 to 90 per cent, and on the fourth count 60 to 75 per cent. Thus the last counts made on the 5th day after the crisis showed the return to an entirely normal range of total white cells with the normal differential both as to supravital and Arneht classifications except for some increase in the monocytes, 8 to 14 per cent. Hickling (15) has just made a very interesting analysis of the monocytes in pneumonia, correlating their late rise in the course of the disease with resolution, and drawing certain prognostic conclusions from their curve from day to day.

Such an analysis, as here attempted, of successive white blood cell counts during a leucocytosis might indicate a periodic delivery of new cells from the hemopoietic organs, the intervening intervals of decreasing total circulating neutrophils being representative of their constant loss to the tissues at the site of the disease in their functional capacity of local cellular defense. The importance to the surgeon of the recognition of these rapid fluctuations in cases of an acute abdominal condition, with the advisability of frequently repeated counts when the diagnosis is in doubt, is evident. The degree to which the multiplication and maturation, of the particular cell type required for the body defense in a specific disease, may be speeded up is an approximate index of the cellular factor in the complex of the "resistance" of that individual (16, 17).

### *Lymphocytosis.*

Case 14 (Chart 12A) presented an equivocal picture on admission to the Hospital with few symptoms, a low grade fever, a cervical adenopathy, and a moderate leucocytosis. The differential count showing a decided lymphocytosis was suggestive and the eventual development of the characteristic cough established the diagnosis. The 3 hour period of 15 minute counts in this case of pertussis reveals a curve of fluctuations in the lymphocytes quite distinct from that of the leucocytes. Because the lymphocytes are in total numbers actually in excess of the neutrophils the fluctuations in the former tend to be reflected in the total count. It will be seen that the usual hourly rhythm of the myeloid cells is maintained, that the peaks are made largely by the influx of young cells (Chart 12B), and that the lymphocytes fluctuate with each count, or on a half hourly swing, as was found for the lymphocytes in normal counts (1). The monocytes, eosinophils, and basophils are all within normal limits. 1 month after the first series of counts a second period of observation was made just prior to the patient's discharge from the Hospital. It will be noted that the total count had subsided to within normal limits, the lymphocytes having returned to normal percentage and number, and

the neutrophils once more reflect their normal predominance in the curve of the total cells.

Chart 10 is presented in further support of the independence, observed so frequently, in the specific response of different cell strains. The leucocytosis following the administration of adrenalin is essentially a lymphocytosis in which the rise is first of the large young forms and then in the mature intermediate and small lymphocytes (10B). This curve is representative of three similar cases, one being a normal individual. It would appear that the change in the white cells under these conditions is not the reflection primarily of a blood volume or redistribution phenomenon, but rather the result of a direct influence on the lymphocyte supply, or its physiological control. Rous (10) found in dogs that the tissues producing lymphocytes are "set" at a rate of activity definite for the individual but subject to wide variations under the influence of changing physiological conditions. Inasmuch as such physiological stimuli are always potentially interactive, in the normally functioning individual periodicity in the delivery of lymphocytes may be assumed on the basis of the experimental work.

It is difficult to escape the impression that there are independent physiological controls for maintaining the relative, normal levels of the different types of white blood cells (14, 18), just as there is a body, growth-limiting, mechanism, a heat-regulating center or mechanism, and a cardiac regulatory mechanism. The readjustment of the control of the normal level for a specific cell strain, while not influenced appreciably by body posture, vasomotor instability, or variation in local distribution (19, 20), nevertheless must be subject to many chemical and toxic influences (12, 16, 21, 22, 23), and may be independent of the actual supply of the particular cells, as was indicated in the case of Minot and Isaacs (24) in which, following a large transfusion of lymphoid leucemic blood into a patient with marked lymphopenia, the original low level of lymphocytes was regained within  $2\frac{1}{2}$  hours without evidence of cell destruction and without constitutional reaction. This observation indicates an altered regulatory mechanism in this patient for maintaining a low level of circulating lymphocytes independent of the supply or of a primary lymphocytolytic factor. However, in the individual physiological mechanism, it is probable that the altered control factors are

usually interactive with the formation and maturation processes for the particular cells affected and respond to the general law of supply and demand. Obviously the above discussion does not relate to true cytolytic effects on the mature circulating cells of any strain, the reaction to which is usually a compensatory hyperplasia in the attempt to establish an equilibrium at the new level of turnover in the particular cell type, and is simply the effort to maintain the normal established level for the physiological functioning of that cell.

### *The Leucemias.*

When a study of the variations in the peripheral blood in the leucemias is attempted, however, it appears that the laws which seem to govern formation, maturation, and delivery of the cells involved, under normal and other pathological conditions, no longer hold. No evidence in Case 19 (lymphoid leukemia), or in Case 20 (myeloid leukemia), of the periodic rhythmicity more or less characteristic of the curves of lymphocytic and myeloid cells discussed above, can be found, as perhaps could hardly be expected when the presence of large numbers of immature forms in the peripheral blood indicates an entirely disordered mechanism of maturation and delivery.

The 3 hour period of observation in the case of chronic lymphoid leukemia cutis gave a range of fluctuation in the total count from 35,000 to 52,000, and the lack of conformity in the curves of lymphocytes and neutrophils and the unaffected status of the monocytes can be seen (Chart 17A). However, it is impossible to establish any periodicity in the total lymphocyte curve within the time period recorded. Charts 17D and E indicate a relative relationship between the young immature cells and the total lymphocyte curve, both falling during the first 2 hours, and Chart 17D, compiled from the supravital differential counts, shows the large number of lymphocytes showing normal motility. The neutrophils maintain an undisturbed hourly elevation and fall (Chart 17A), and show an Arneith differential count within normal limits (Chart 17C). The increased eosinophils in this case are shown on Chart 17B, the rhythm being an hourly one though independent of the neutrophil fluctuations. The eosinophilia in the case of erythrodermia (Chart 6) is quite comparable and probably this specific response in both cases is related to the respective skin involvements. Cioni (25) discusses the mechanism of eosinophilia relative to the nature of the specific chemical stimuli altering the bone marrow supply of these cells, and believes that in the case of infectious skin conditions it may be due either to the metabolic products of the parasite or to products of the destruction of the epithelium, or to both.

The striking thing in the analysis of the fluctuations in the case of myeloid leucemia, with a range in the total count from 200,000 to 284,000 during the 3 hour period of observation, was the inverse relationship between the mature neutrophils and the myelocytes (Chart 18A). Since it was a chronic case, the myeloblasts were relatively few in all counts. With every increase in the number of mature leucocytes there was a corresponding fall in the myelocyte level, and *vice versa* (26). This was particularly striking in the curves of neutrophilic myelocytes and the young mature neutrophils (Chart 18B), an increasing divergence in these two curves being manifest toward the end of the period, the later total counts reflecting this rise in the myelocytes directly. The independent fluctuations of eosinophil, basophil, and neutrophil myelocytes show only the disordered chaos of the myeloid hyperplasia of the bone marrow, though the mature cells still show some semblance of a periodic fluctuation with three peaks during the 3 hours. In the leucemias it would seem that all regulation both as to primary formation and delivery and as to concentration of the cells in the peripheral blood was gone.

### *The Bone Marrow.*

Any attempt at a quantitative estimation of the various cellular elements making up the bone marrow must, in the very nature of the survey, always be open to many questionings: the limitations inherent in any technic used, the fallacies inescapable in trying to draw any general deductions from even 1000 or 2000 cells counted out of the multiple millions present in any functioning bone marrow, the much debated questions of identification and classification of immature forms, the many factors known and unknown affecting hemopoiesis in any particular individual, all of these, and more, make it necessary to be very conservative in the drawing of deductions from any one limited series of observations or in trying to compare the figures obtained from different investigators.

Using the supravital and fixed technic as outlined at the beginning of the paper, we have found a reasonable degree of conformity in our counts, and in so far as any constant procedure can assure comparable results, the findings, as contained in Table III, may be so analyzed. Only very general indications, which might be most readily admitted as based on indisputable cellular identifications, will be attempted.

In the six cases under 8 years of age the relatively high percentage of lymphocytes will be noted, with none or very few mature polymorphonuclear leucocytes. This is in conformity with the well recognized relative lymphocytosis in children. In the presence of infection



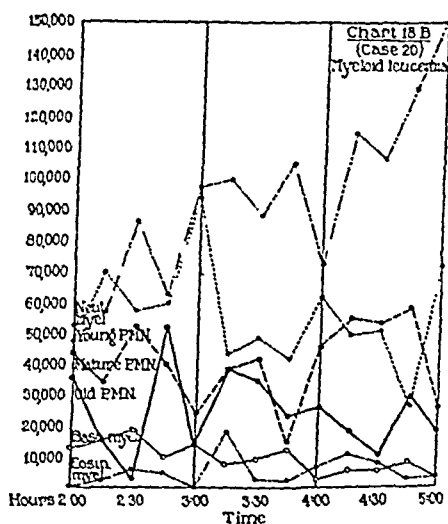
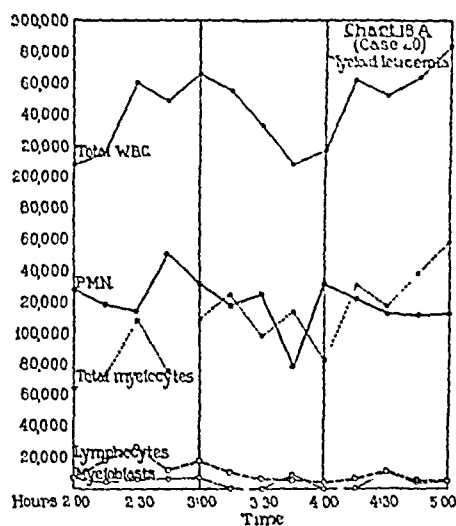
TABLE III.  
*Human Bone Marrow Differentials Based on Count of 1000 to 2000 Cells.*

Diagnosis	Polys.	Myelocytes			Myeloblasts	Prim. cells	Classmateocytes	Monocytes	Megakaryocytes	Lymphocytes	Lymphoblasts	Eosin.	Basoph.	Megakoblasts	Normoblasts	Uncl.	Age
		Neut.	Eosin.	Basoph.													
1. Acute dehydration.....	0.	35.2	1.4	0.	5.9	0.	0.3	9.4	0.4	38.9	0.	2.4	0.	0.1	5.2	0.8	4 mos.
2. ".....	0.	31.1	2.2	0.6	5.	0.4	1.2	4.2	0.2	44.9	0.	0.	0.	0.	9.1	1.1	5
3. Normal (accident).....	15.3	31.	0.37	0.	1.5	0.81	0.27	4.5	0.45	37.3	0.	0.54	0.	0.	5.7	1.1	4 yrs.
4. Diphtheria.....	4.8	56.4	1.6	0.9	1.5	0.2	0.9	3.1	0.6	27.7	0.	0.	0.	0.	1.5	0.8	6
5. Pertussis-bronchopneumonia.....	0.6	61.8	0.6	0.	1.	0.	0.3	2.1	0.4	28.3	0.	0.	0.	0.1	3.5	1.3	8
6. Bronchopneumonia (vertebral bone marrow).....	0.9	56.9	3.	0.1	4.2	0.5	0.6	3.	0.1	29.1	0.	1.	0.	0.	0.6	0.	7
7. Normal (accident).....	55.4	18.8	4.1	0.	1.1	1.1	0.	1.6	0.1	10.8	0.	0.6	0.1	0.1	5.4	0.8	28
8. ".....	10.5	53.1	0.8	0.	11.9	0.4	0.1	3.5	0.	15.	0.	0.2	0.	0.	4.1	0.4	29
9. ".....	13.7	54.9	0.1	0.	1.7	1.	0.	0.5	0.1	14.7	0.	0.	0.	0.	13.	0.3	30
10. Acute cerebral hemorrhage.....	19.7	40.5	0.9	0.3	5.	0.3	1.	5.2	0.5	9.1	0.	0.7	0.	0.	14.2	2.6	72
11. Lobar pneumonia.....	19.2	63.5	0.6	0.	4.6	0.2	0.3	1.1	0.	8.8	0.	0.	0.	0.	1.7	0.	28
12. ".....	90.	5.	0.1	0.	1.9	0.	0.1	2.	0.1	0.	0.	0.8	0.	0.	0.	0.	60
13. Empyema (biopsy rib).....	47.7	30.3	0.8	0.	8.5	0.7	0.2	0.8	0.4	6.3	0.	0.3	0.	0.6	3.	0.4	42
14. Lung abscess.....	46.7	33.3	1.	0.6	2.7	0.4	0.	2.	0.	6.2	0.	0.2	0.	0.8	3.8	0.7	32
15. Subdiaphragmatic abscess.....	65.4	15.	1.7	0.	2.6	0.3	0.	0.9	1.1	7.4	0.	1.7	0.	0.1	3.1	0.7	38
16. Empyema (biopsy rib).....	46.	30.6	1.9	0.	2.6	1.4	0.	1.1	0.2	10.1	0.1	0.3	0.	0.4	4.7	0.6	38
17. Postpartum infection.....	27.8	37.8	0.3	0.	3.5	0.1	0.	6.8	0.4	15.	0.	1.1	0.	0.	6.8	0.4	44
18. General sepsis.....	5.7	65.1	0.3	0.	1.4	0.1	0.1	13.8	0.2	8.3	0.	0.	0.	0.2	4.1	0.3	36
19. Cardiovascular lens. Pneumonia.....	9.3	71.	0.4	0.2	1.1	0.2	0.1	4.7	0.2	9.3	0.	0.	0.	0.	2.1	1.4	59
20. Cirrhosis of liver.....	4.4	75.1	0.5	0.2	2.5	0.6	5.2	3.4	0.7	5.	0.	0.5	0.	0.	1.7	0.2	61
21. ".....	1.6	52.7	1.6	0.5	8.	3.1	0.6	8.1	0.1	3.6	0.	0.1	0.	0.	17.7	3.3	46

22. Cirrhosis of liver .....	6.	58.7	1.1	0.	0.	1.3	0.7	8.70	0.4	20.3	0.	0.1	0.	0.8	0.	57	
23. " " (infection) .....	26.3	50.1	0.6	0.1	2.1	1.	1.4	1.30.	13.1	0.	0.6	0.	0.	3.	0.4	49	
24. Pernicious anemia .....	7.6	62.9	1.7	0.1	4.7	0.6	5.5	6.80	0.6	5.8	0.	0.5	0.	0.6	1.	62	
25. " " .....	2.9	54.6	4.1	2.6	0.3	0.7	5.5	4.30.	20.1	0.	1.5	0.2	1.9	1.	0.5	61	
26. " " .....	0.5	14.8	30.3	0.9	5.3	0.	6.7	12.60.	10.3	0.	0.	0.	10.6	3.2	0.8	56	
27. " " .....	6.8	56.	1.6	0.2	1.6	0.5	5.6	7.10.	16.8	0.	0.7	0.	1.5	1	0.6	58	
28. " " .....	8.1	12.8	10.6	1.6	19.7	0.8	3.4	13.10.	11.6	0.	0.6	0.2	14.4	2.1	1.	46.	
29. Acute miliary tuberculosis .....	31.1	9.42	0.38	0.09	2.66	0.19	0.	21.90.	32.	0.	0.66	0.	0.	1.42	0.57	36	
30. Lymphatic leucemia. Cirrhosis of liver.....	0.	0.	0.	0.	0.	0.	0.	4.80.	0.	93.	0.	0.	0.	0.4	1.8	48	
31. Banti's disease.....	5.1	42.4	2.2	0.	2.	0.3	8.2	5.60	0.3	27.6	1.3	0.8	0.	0.3	2.8	1.1	48
32. Carcinoma of pleura.....	7.3	42.	14.7	0.9	3.	0.3	5.2	1.30	0.5	13.6	0.	1.8	0.	0.	5.4	3.	52

(Cases 4, 5, and 6) the myeloid cells were increased though the response was not to the extent seen in adult cases (27).

There are four cases in the series (Cases 3, 7, 8, and 9) which have been considered to present normal bone marrow pictures, inasmuch as they were medicolegal cases of sudden accidental death in supposed healthy young adults (one child of 4 years). It is only desired to call attention to the relationships found between mature polymorphonuclear neutrophilic leucocytes and the neutrophilic myelocytes. It may be granted that the distinction between cells with characteristic multilobed nuclei and their full complement of specific granules and those with oval single nuclei and relatively fewer specific granules is



possible of ready appraisal by any worker familiar with the blood. Thus while the total myeloid percentage in the three adult cases showed close agreement, 74 per cent, 64 per cent, and 68 per cent, in Case 7 there were 55 per cent segmented forms and 19 per cent myelocytes, in contrast to Cases 8 and 9 with only 10 and 13 per cent mature cells and 53 and 55 per cent myelocytes, respectively. Schilling (28) and Benzler report a cytological study of the bone marrow from ten normal healthy men, just shot, in which the myeloid elements ranged from 39 to 43 per cent, eosinophils from 1.1 to 4.6 per cent, and normoblasts from 31 to 42 per cent. The peripheral blood in these cases showed neutrophils ranging from 32 to 74.5 per cent, and

in the bone marrow differentials, the myelocytes varied between 34 and 47 per cent, the metamyelocytes between 34 and 56 per cent, and the segmented nuclear cells between 5 and 22 per cent, but there is no relationship expressed in the individual cases. In the present series, with the technics used, normoblasts were found in much smaller relative percentages than those given above by Schilling, *viz.* 4 to 13 per cent in the normal cases and from 0 to 17.7 per cent in the pathological cases. The latter figures are more in accord with those reported in a small series of pathological cases by Doan (7) with the supravital technic, in which the normoblasts were found in percentages of from 4 to 22. A study of the fixed sections of bone marrow, except perhaps in certain severe secondary anemias and pernicious anemia, gives a definite impression of the predominance of myeloid metaplasia over erythroid foci in contrast to the relative numbers of the respective definitive cells in the peripheral blood. This must be related to the more rapid turnover in the white cells, the red cells under most conditions probably surviving for a much longer period; also the entire functional activity of the red cells is dependent upon their circulation within the vascular bed, whereas the circulation is simply the means of communication for the white cells from source of supply to the local points of activity in the body outside the blood stream. Certainly with the supravital technic, which has now been used in many scores of surveys of bone marrow from experimental animals (29, 30), and with the fixed technic as here used, the nucleated red cells have been far more often below 20 per cent of the differential than above.

In two instances of lobar pneumonia (Cases 11 and 12) the total myeloid cells in the bone marrow were 82.7 and 95 per cent respectively, but in the former there were 19.2 per cent mature cells and 63.5 per cent myelocytes, whereas in the latter the mature cells were 90 per cent and myelocytes 5 per cent.

A comparison of the first two columns of Table III straight through shows the reciprocal relationship maintained between the percentages of mature and immature neutrophils. When taken into account in the presence of the observed periodic fluctuations in the total white blood count in the peripheral blood, as presented in the first part of this paper, a tenable hypothesis of the periodic maturation of myelo-

cytes into polylobed, motile leucocytes may be conceived. The particular moment at which the process of manufacture is stopped would give for a given area either a majority of mature neutrophils or the reverse according to the phase of maturation at that point at that time. This has been particularly striking in the experimental study of the bone marrow after specific stimulation of the maturation of the neutrophil group by sodium nucleinate and similar substances producing a peripheral leucocytosis (29). In bone marrows relatively hypoplastic, so that the two processes of red and white cell formation may be analyzed separately, it is striking to find always the cells in any one focus or area all at the same stage of maturation (30, 31, 32). Thus, it would seem from all the evidence at hand that the white cells, leucocytes, lymphocytes (33), and monocytes (34, Simpson "macrophage showers") are constantly leaving the blood stream, and that the supply is primarily dependent upon a more or less periodic maturation and delivery of granulocytes from the bone marrow, lymphocytes from the thoracic duct (10, 35), and monocytes and clasmatoocytes possibly from the spleen. It is not believed that other factors are negligible as affecting the total numbers of circulating cells, but that the factors mentioned are important in the cellular economy of the body seems probable.

In view of the recent attention attracted to the cellular aspects of experimental and clinical tuberculosis (36, 37) it is of interest to note the one case (No. 29) of miliary tuberculosis in which the monocytes in the bone marrow were 21.9 per cent and the lymphocytes 32 per cent. It has been shown (36) that the typical epithelioid cell of tuberculosis, when stained with one of the Romanowski stains, reacts with a characteristic faint eosin tint in the cytoplasm near the *Hof* of the nucleus where the rosette of neutral red bodies is found in the supravital preparation. In this case these cells could not be mistaken, and though listed as monocytes the majority of the cells were true epithelioids. In no other case were monocytes found in as high percentages in the bone marrow, and the lymphocytes only in the cases within the first decade of life.

The five cases of pernicious anemia, Nos. 24, 25, 26, 27, and 28, show the well recognized (7, 38) increased percentages of clasmato-

cytes, again a cell type easily recognized through its marked capacity for phagocytosis.

#### SUMMARY.

In a study of twenty clinical cases with a wide range of diagnoses, repeated total counts of the white cells at 15 minute intervals reveal a large fluctuation at various levels comparable to that found for the normal (1, 2). The granulocytes seem to follow a more or less hourly rhythm, the most marked shift to the left in the Arneth pattern and the moment of greatest percentage of motility coinciding with the peaks.

The independence found existing between the peripheral blood concentrations of individual strains of white cells and the red cells, as determined by total and differential counts, their differential response to pathological and pharmacological stimuli, and their normal relative relations, all indicate some separate physiological mechanism of control for each type of cell, either working through, or independently of, their sources of origin.

The many factors to which the circulation of the blood, as such, is subject, the complexity of the influences on origin, maturation, delivery, longevity, and destruction of each cell group, the limitations inherent in the present involved, indirect technics of counting, combine to make any single observation subject to grave misinterpretation. The value to the clinician must come in repeated observations, at times when the diagnosis or a therapeutic procedure is in doubt, at frequent intervals, at other times over longer or shorter periods, but always with the relation between consecutive counts, rather than the absolute values, the important point for consideration.

Both the red and the white cells probably change their relative concentrations in the peripheral blood from time to time over a considerable range that is quite within normal physiological limits, so that, in theoretical considerations and in practical functional estimations, a zonal concept with adequate individual extremes should always be kept in mind for both physiological and pathological states.

A cytological analysis of thirty-two bone marrows from human biopsy and autopsy material shows the striking reciprocity found to

exist between the myelocytes and the mature polymorphonuclear leucocytes. This, together with the observed focal uniformity of maturation found in bone marrow, and the periodicity of the fluctuations of the neutrophils in the peripheral blood, leads to the formulation of the hypothesis of a constant functional withdrawal of granulocytes from the peripheral blood with a periodic delivery of new cells from the marrow, which in leucopenia and in leucocytosis represents a depression or a stimulation, respectively, of the normal mechanism. The nature and degree of the response are an approximate index of the cellular factor in the complex of the "resistance" of the particular individual.

It is a privilege and a pleasure to acknowledge here the constant interest and counsel of Dr. Francis W. Peabody and the assistance of the following individuals in particular phases of this work: Dr. Alberto Hurtado in the cases of acute rheumatic fever; Dr. Perrin H. Long in the observations with adrenalin; Dr. Emil A. Falk in the clinical study of Cases 2 and 18; and Miss Sylvia Warren and Miss Olivia Ames in the studies on Case 19.

#### BIBLIOGRAPHY.

1. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 14.
2. Shaw, A. F. B., *J. Path. and Bact.*, 1927, xxx, 1.
3. Bushnell, L. D., and Bangs, E. F., *J. Infect. Dis.*, 1926, xxxix, 291.
4. Van Allen, C. M., *J. Am. Med. Assn.*, 1925, lxxxiv, 202.
5. Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 277.
6. Sabin, F. R., and Doan, C. A., *J. Exp. Med.*, 1927, xlvi, (in press).
7. Doan, C. A., *J. Exp. Med.*, 1926, xliii, 289.
8. Howe, P. E., *Physiol. Rev.*, 1925, v, 439.
9. Barcroft, J., *Lancet*, 1925, i, 319.
10. Rous, P., *J. Exp. Med.*, 1908, x, 238.
11. Swift, H. F., Miller, C. P., Jr., and Boots, R. H., *J. Clin. Inv.*, 1924, i, 197.
12. Hickling, R. A., *Folia haematol.*, 1927, xxxiii, 199.
13. Pons, C. A., and Ward, E. P., *J. Lab. and Clin. Med.*, 1925, xi, 103.
14. Mauriac, P., and Moureau, M., *J. méd. Bordeaux*, 1923, xcv, 39.
15. Hickling, R. A., *Arch. Int. Med.*, 1927, xl, (in press).
16. Ferguson, B., *Med. J. and Rec.*, 1924, cxx, 580.
17. Jones, W. C., and Crocker, F. L., *J. Lab. and Clin. Med.*, 1927, xii, 482.
18. Schilling, V., *Deutsch. med. Woch.*, 1925, li, 516.

19. Ziegler, K., *Klin. Woch.*, 1924, iii, 1481.
20. Liebenstein, A. F. V., *Klin. Woch.*, 1924, iii, 1482.
21. Feringa, K. J., On the causes of emigration of leukocytes, Inaugural dissertation, from the Physiological Laboratory of Gröningen State-University, Feb. 23, 1922.
22. Bacon, D. K., Novy, F. C., and Eppler, H. H., *Arch. Int. Med.*, 1922, xxx, 229.
23. Howery, G. G., *Virginia Med. Month.*, 1925, lii, 313.
24. Minot, G. R., and Isaacs, R., *J. Am. Med. Assn.*, 1925, lxxxiv, 1713.
25. Cioni, G., *Polidlinico, sez. prat.*, Rome, 1925, xxxii, 1041.
26. Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A., *J. Exp. Med.*, 1924, xl, 845.
27. Lateiner-Mayerhofer, M., *Z. Kinderheilk.*, 1914, x, 152.
28. Schilling, V., *Deutsch. med. Woch.*, 1925, li, 344.
29. Doan, C. A., unpublished experiments.
30. Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
31. Doan, C. A., and Sabin, F. R., *J. Exp. Med.*, 1927, xlvi, 315.
32. Peabody, F. W., *Am. J. Path.*, 1926, ii, 487.
33. Bunting, C. H., and Huston, J., *J. Exp. Med.*, 1921, xxxiii, 593.
34. Simpson, M. E., *Univ. Calif., Pub. Anat.*, 1921, i, 11.
35. Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1927, xl, 39.
36. Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxvii, 231.
37. Cunningham, R. S., and Tompkins, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xiv, 695.
38. Peabody, F. W., and Broun, G. O., *Am. J. Path.*, 1925, i, 169.